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(54) Title: METHOD FOR IDENTIFYING AND CONFIRMING CONSISTENT BIO–FUNCTIONALITY OF NATURAL COMPOSITIONS

(57) Abstract

The present invention provides methods for evaluating the biofunctionality of natural products. Since natural products typically comprise a variety of active components, both identified and unidentified, and normal dosage is by ingestion, methods of the invention simulate normal physiological digestion and absorption from the digestive tract. Methods are disclosed which provide for evaluation of biofunctionality and quality assurance testing for natural compositions such as herbs, herbal extracts and formulations derived therefrom, even though active components and their specific activities may remain unidentified.

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METHOD FOR IDENTIFYING AND CONFIRMING CONSISTENT BIO-FUNCTIONALITY OF NATURAL COMPOSITIONS

This application claims the benefit of U.S. Provisional application Ser. No. 60/092,686 filed 14 July 1998 and of U.S. Provisional application Ser. No. 60/094,360 filed 28 July 1998.

FIELD OF THE INVENTION

The present invention relates to a method for assessing the functional biological activity of natural products in the forms of raw material and as finished products as consumed, and the natural products subjected to such method.

BACKGROUND OF THE INVENTION

Natural products that are used as dietary supplements are not typically considered to be drugs, yet the consumer frequently expects many of the same properties, efficacy and safety, from natural products that are expected of drugs. Pharmaceutical agents (drugs) designed for specific treatment of disease are regulated by the Food and Drug Administration (FDA) and consist of a single or limited and defined mixture of active ingredients. Once a drug compound has been selected, formulated, and then proven safe and efficacious for the disease aimed to be treated as designated by FDA guidelines, the product needs only to be assayed only to assure proper content of the active compound. Effectiveness to the physician and consumer is then governed by the history of the compounds proven clinical effectiveness.

Unlike pharmaceutical agents, assessment and standardization of natural compositions is often problematic. The components may be difficult to assay and are often too numerous to standardize with statistical confidence. In addition, the chemical compositional makeup of a natural product will also depend upon where and how the material was grown, harvested and processed, as well as environmental conditions such as nutrients, diseases and insect damage. Furthermore, the active ingredients may be unknown and thus standardization for one type of chemical entity may be detrimental for activity or the proper ratios for presumed efficacy of multiple active ingredients may be unknown.

The market for natural products and other dietary supplements has evolved in an environment of traditional analytical quality assurance techniques based on standardization of the natural raw materials. As alluded to above, this strategy to ensure quality and consistency does not ensure consistent biological effect of the type promoted via claims on product labels. For example, the implied quality of a popular product known as St. John's Wort is based on standardization of a single chemical entity (hypericin), which may not be responsible for the benefits for which St. John's Wort is sold.

In the United States, natural products are frequently characterized to the consumer by nonspecific statements of nutritional support and general well being as permitted under the Dietary Supplement Health and Education Act (DSHEA) such as "antioxidants maintain cell integrity." This type of claim is meant to inform consumers of potential benefits in maintaining normal bodily structure and function without making direct claims for treatment of specific health problems such as would necessitate regulation under the Federal Food, Drug and Cosmetic Act (FFDCA).

As claims for these products are becoming both more aggressive and correspondingly more carefully scrutinized by consumers and regulatory agencies, it is increasingly important that quality assurance methods be developed to validate those claims and to differentiate to the public products which do not provide their claimed benefits from those that do.

Development and utilization of biological assays designed to assess the efficacy of natural products in providing their claimed benefits provides a means to evaluate quality and consistency of materials which contain amounts of chemical constituents which can vary greatly from preparation to preparation. In addition, unlike pharmaceuticals which are traditionally designed for specificity and potency via a single mode of action, natural products tend to have multiple sites or modes of action, which when combined, produce the intended benefit.

The present invention is directed toward addressing the above-noted problems related to assuring consistent levels of biological activity corresponding to the functional use of the natural products.

SUMMARY OF THE INVENTION

The present invention employs a functional approach to testing and assessing the quality of the complex mixtures which characterize natural compositions.

The present invention relates to a method for evaluating the biofunctionality of a natural composition or extract which comprises subjecting the natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said natural composition, determining the presence or absence of one or more components or biological activities in said preparation, and correlating the presence or absence of said one or more components or biological activities in said preparation to the biofunctionality of said natural composition or extract. Simulated digestion and simulated absorption can be carried out sequentially or concurrently.

Simulated digestion can be simulated gastric digestion or simulated intestinal digestion or can be a combination of both. Simulated digestion is performed in a gastric fluid which is acidic, preferably around pH 1.2, and which may also comprise digestive enzymes which are active under acidic conditions or in an intestinal fluid which is neutral, preferably about pH 7.4 which may also comprise digestive enzymes which are active under neutral conditions.

Simulated absorption involves transport of components or biological activities across a membrane for the purpose of simulating bioavailability. In the preferred embodiment, the membrane is a monolayer of Caco-2 cells. It is an object of the present invention to provide a system of assays for any given natural composition wherein the system is specifically tailored to assess the relevant biofunctionality of that composition. The present invention provides assay methods that assess the expected *in vivo* biofunctionality of a natural composition and thus, assess the usefulness of the natural composition for treatment of a selected pathological

condition or as a dietary supplement for human consumption useful in maintaining healthy structure and function. Useful assays can also include assays which define or measure undesired side effects that may be associated with a natural composition.

The invention is further directed to a dietary supplement comprising a natural composition, having substantial batch-to-batch compositional consistency, said compositional consistency evaluated by a method comprising subjecting a sample of the dietary supplement or the natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said sample, determining the presence of one or more components in said preparation, and correlating the presence of one or more components with compositional consistency.

By compositional consistency is meant substantial uniform batch-to-batch biofunctionality of at least one desired active component or biological activity.

Another aspect of the invention is a system of testing which is useful for the establishment of standards for natural compositions as well as for assuring the quality and consistency of natural dietary supplements. Rather than attempting only to identify and quantify the active components of any given complex natural composition as consumed, the system of testing is designed to assess the overall biofunctionality of that composition as consumed, and relies on tests which are specifically tailored to ensure efficacy and consistency for corresponding natural products.

The invention is further directed to a process for making a dietary supplement comprising a natural composition which has substantial batch-to-batch consistency, said compositional consistency evaluated by a method comprising subjecting a sample of said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said sample, determining the presence or absence of one or more components in the preparation, and selecting preparations comprising said one or more components.

The invention also relates to a novel method for evaluating the biological activity of a *Ginkgo biloba* composition which comprises subjecting a neuronal cell in the presence of said composition to one or more substances or conditions effective to reduce viability of said neuronal cell and measuring the viability of the neuronal cell.

Another aspect of the invention is a novel method for evaluating the biological activity of a saw palmetto composition which comprises detecting inhibition of the transformation of androgen response receptor to DNA binding form by providing a mixture containing said composition, 5HT, unliganded androgen receptor complexed to HSP90 and androgen response element DNA, and detecting the formation of an androgen receptor-androgen response element DNA complex.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the effect of sample preparation method on the macrophage activation capacity of an *Echinacea* reference product. Data represents the mean ± standard deviation of TNF-α produced from the RAW264.7 macrophage cell-line treated with 20 μg/ml *Echinacea purpurea* herb from different sample preparations. E-DMSO is the reference product capsule contents dissolved in dimethylsulfoxide (DMSO) solvent; E-50% ETOH is the reference product capsule contents dissolved in a 50% Ethanol/water solution; E-Digestion is the reference product capsules subjected to a simulated digestion protocol. Placebo is placebo soft gel capsules provided by R.P. Scherer NA.

Fig. 2 depicts the dose-response for macrophage activating capacity of an *Echinacea* reference product for a preparation subjected to simulated digestion. Data represent the mean of three replicate wells. Standard deviation was less than 5%.

Fig. 3 depicts the macrophage activating capacity of seven *Echinacea* raw material samples as compared to two reference standards and a placebo control following simulated digestion. Data represent the mean ± standard deviation for three replicate wells.

Fig. 4 depicts the macrophage activating capacity (corrected for placebo activity) of eleven *Echinacea* product samples as compared to a reference standard following simulated digestion. Data represent the mean ± standard deviation for three replicate wells.

Fig. 5 depicts the results of an assay assessing functional bioavailability for samples of an *Echinacea* reference product and a placebo. Macrophage activating activity was determined before and after simulated absorption by incubation with a Caco-2 cell monolayer. The bars represent the mean ± standard deviation for three replicate assay wells.

Fig. 6 depicts the lot-to-lot variation for five lots of an *Echinacea* reference product. Data represent the mean ± standard deviation for three replicate wells.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention will now be described in detail for specific representative embodiments of the invention, it being understood that these embodiments are intended only as illustrative examples and the invention is not to be limited thereto.

For the representative embodiments specifically disclosed herein, the term natural composition is used to refer to a composition believed to be useful as a dietary supplement for maintaining normal structure and function for general well being as defined in DSHEA. A natural composition is preferably from a botanical source, and more preferably from an herbal source, but can also be from sources that are not botanical, for example, compositions from animals and other non-botanical living organisms, such as, for example, algae, fungi, bacteria and the like. In their least processed form, natural compositions include the source itself, as well as selected parts of the source. Botanical and herbal compositions can include, for example, stems, flowers, fruits, roots, etc. Leaves of a plant which have been packaged, whole or ground up, for brewing tea, for example, are natural compositions. Natural compositions include extracts from the source, for example, ingredients separated from the source by solvent extraction. Solvents can include water, ethanol, methanol, acetone and the like. Other examples of solvents employed in the making of extracts are well know to one of average skill in the art. Natural compositions can also include materials from the source

which have been modified by other processes such as, for example, enzymatic or chemical modification or digestion. Natural compositions are compositions which comprise all or part of a botanical, herbal, animal or other living organism, and can be unprocessed or processed by physical, enzymatic or chemical means. Natural compositions also include such compositions to which have been added other substances. Other substances are those added for example, to preserve or formulate preparations of the natural compositions, and include for example, preservatives, fillers, binders, lubricants and the like. Natural products are comprised of natural compositions that are in a form ready for use or consumption.

In contrast with a pharmaceutical composition that is typically comprised of a single active compound, a natural composition is comprised of a mixture of potentially active and interactive constituents. Such a mixture may contain only two or three constituents that have been identified as being primarily responsible for producing the desired biological effect, but typically contains many more. In addition, when prepared as an extract or mixture of extracts from naturally occurring substances, e.g. substances found in living organisms, a natural composition typically contains many additional constituents, which may be individually present in relatively small and varying quantities, but which in combination may constitute a significant fraction of the total natural composition. These additional secondary ingredients may synergistically contribute to the effectiveness of the primary active ingredients in producing the desired biological effect as well as in suppressing undesired side effects.

Although it is well known that the bioavailability and, therefore, efficacy of a biologically active substance can be dramatically affected by other substances which may be co-administered, a natural composition differs from co-administered pharmaceutical compositions, *inter alia*, in that a natural composition is extracted from naturally occurring substances in a manner that results in a composition containing a mixture of both primary and secondary constituents.

Biofunctionality, as used herein, means the capacity of a composition to bring about an overall change in a condition or characteristic of a living organism. For a composition of natural origin, biofunctionality means the capacity to affect some aspect of health or well

being when consumed as a medicament or dietary supplement. Typically the overall benefits to health and well being of a natural composition are determined by clinical trials that test effectiveness for the claimed use in treating a health related condition or disease. Claimed effects of natural compositions can include, but are not limited to, boosting of the immune system when it is under high stress and enhanced wound healing (*Echinacea*), maintenance of prostate health (Saw Palmetto), enhanced blood circulation to the extremities and the brain which improves mental capacity (*Ginkgo biloba*), improvement of liver function and protection against liver damage (*Silymarin*), and as a circulatory stimulant and anti-nauseant (Ginger root). Clinical trials typically are not done in a prospective manner due to high cost. In addition, effects attributed to natural compositions such as maintenance of health and well being and prevention of a disease or an undesired condition cannot be tested by established clinical trials.

Biological activity of a composition means the capacity of a composition to affect a specific biological characteristic of a cell or group of cells in an *ex vivo* assay or a biochemical reaction by which it is being tested. Such characteristics include, but are not limited to changes in visible phenotypes, alterations in patterns of gene expression (i.e., alteration in levels of specific mRNA and proteins or polypeptides), changes in secretion of molecules capable of affecting other physiological processes (e.g., cytokines, interleukins, histamine) or changes in sensitivity of test cells to chemicals with known effects (i.e., responsiveness of cells to external stimuli). The efficacy of a particular natural composition for achieving the desired biofunctional effect may thus be assessed by testing certain specific biological activities that are associated with that particular biofunctional benefit.

Because the ingested natural composition typically contains a mixture of potentially active constituents, and because the range in biological activity may vary considerably depending on the actual *in vivo* availability (i.e., bioavailability) of each constituent in the natural composition, it is important that assessments of biofunctionality include assays that reliably predict what the biological activity of that natural composition will be *in vivo*. The present invention, thus, provides a unique system for reliably predicting the *in vivo* efficacy of a natural composition on the basis of how it performs in a variety of functional tests once the

natural composition has been subjected to simulated digestion and/or absorption. The tests are conducted in the form of specifically selected bioassays that demonstrate *in vitro* biological activities that are closely associated with the *in vivo* biofunctional benefits ascribed to the natural compositions and often established via clinical trials and subsequent mechanistic studies. Furthermore, these assays have been validated for specificity of response and consistent performance in order to provide reliable results.

In addition to selecting assays for a given natural product, sample preparation methods are employed to achieve the greatest possible correlation with *in vivo* activity in humans. Such sample preparation may include the use of a simulated digestive model. Simulated digestion may include simulated gastric digestion, simulated intestinal digestion, simulated oral digestion, or a combination thereof. In particular, in order to assess the bioavailability of the active components of the natural composition, assay methods of the present invention may employ sample preparation methods which simulate aspects of passage of the natural composition through the digestive tract, extending from the mouth to the colon.

As indicated elsewhere herein, passage through the digestive tract can have a significant effect on the bioactive components of a given natural composition. Activity may be reduced or destroyed by digestive mechanisms. Alternatively, those mechanisms may provide for release or activation of inactive precursors from a natural composition. Formulations of natural extracts may be devised which protect components which would otherwise be destroyed by digestion in the stomach. For example, it is well known in the pharmaceutical arts to utilize formulations which are relatively insoluble in the acidic environment of the stomach, but which readily dissolve in the neutral environment of the intestines.

Simulated digestion thus means a process designed to simulate aspects of the passage of natural compositions through the digestive tract. Digestion can be gastric digestion, intestinal digestion or both. To accomplish this, the present invention typically employs a simulated gastric fluid or a simulated intestinal fluid or a simulated salivary fluid. Thus, components of the natural composition are subjected to processes which include inactivation by degradation,

activation through modification, and selection whereby some components may be actively absorbed whereas others diffuse passively or are not absorbed at all.

Gastric digestion typically means subjecting material to be tested to a fluid having a pH below about 2.0 and containing digestive enzymes such as pepsin which are active at acidic pH. Gastric digestion can also include agitation which aids in mixing of fluid and materials so as to simulate the mixing that occurs in the stomach. Preferably acidity ranges from about pH 1.0 to about pH 2.0, and is preferably about pH 1.2. Simulated gastric fluids of the present invention can contain gastric enzymes, most preferably pepsin. Most preferably, a gastric fluid is prepared according to the method of United States Pharmacopoeia (USP) volume 23 and contains pepsin. Natural compositions are typically contacted with such gastric fluids at about 25-40°C, preferably at about 37°C. Gastric digestion is performed for an amount of time which is effective to digest the natural composition or extract. Digestion can be form about 2 minutes to about 10 hours. More preferably, digestion is from about 30 minutes to three hours. Most preferably, digestion is for about two hours.

Intestinal digestion involves neutralization of the acidity of a mixture being digested as it moves out of the stomach into the intestine. Simulated intestinal fluids of the present invention range between about pH 6.0 and about pH 8.0, preferably between about pH 6.5 and about pH 7.5. Such fluids can also comprise digestive enzymes secreted from the pancreas or intestinal lining. Examples of such enzymes are pancreatin, trypsin, chymotrypsin, carboxypeptidase, elastase, lipase and aminopeptidase. Intestinal digestion occurs at a neutral pH and preferably involves agitation that aids in mixing of fluid and materials in a manner similar to that which occurs in the intestines. Intestinal digestion is from about pH 6.5 to about pH 8.0. More preferably gastric digestion is from pH 7.2 to pH 7.6. Most preferably, gastric digestion is about pH 7.4. Gastric digestion is performed for an amount of time which is effective to digest the natural composition of extract. Digestion can be form about 2 minutes to about 10 hours. More preferably, digestion is from about 30 minutes to three hours. Most preferably, digestion is for about two hours.

In another embodiment of the invention simulated absorption comprises transport of one or more components of a natural composition across a biological membrane. Absorption from the digestive tract occurs through the layer of epithelial cells that form the lining of the digestive tract. For example, amino acids and monosaccharides are absorbed across the epithelial membrane and into capillaries by active transport. The epithelial membrane also allows passage of certain substances by diffusion, and can exclude other substances by similar transport mechanisms. Absorption from the digestive tract occurs throughout its length, including intestinal absorption, gastric absorption, sublingual absorption and oral mucosal absorption. The present invention can employ a simulated absorption model that simulates transport of components of natural compositions from the environment of the digestive tract across cells which line the digestive tract and into body fluids and tissues. In a representative embodiment of the present invention, cell monolayers are utilized to simulate physiological absorption. Such cell monolayers are established on a permeable surface, such that both surfaces of the monolayer are accessible to a culture medium, the cell monolayer forming a barrier such that the media on both sides of the monolayer are physically separated. The cell monolayer performs transport functions, simulating absorption from the digestive tract. Thus, simulated absorption comprises transport of individual components of a natural composition from the apical side to the basolateral side of the cell monolayer.

In some cases, where an embodiment of the invention involves the use of simulated digestion which is not incompatible with simulated absorption (e.g., simulated digestion conditions are not harmful to a cell monolayer or other membrane employed for simulated absorption), simulated digestion and simulated absorption may be performed concurrently. In other cases, sequential steps will be required where the simulated digestion conditions are incompatible with the simulated absorption conditions. Performing steps concurrently means that the performance of a digestion step and an absorption step overlap to some degree. It is not required that digestion and absorption of a component occur simultaneously.

Representative cells which may be used to establish a monolayer for the purpose of simulated absorption according to the present invention include Caco-2 cells. Other cells which can be used include, but are not limited to HT29 human colon carcinoma cells and Madin-Darby

canine kidney (MDCK) cells. When plated on a surface which allows the flow of material from apical to basolateral and vice versa, such cells form a biological membrane which can be used to simulate physiological absorption and bio-availability. By biological membrane is meant a structure, such as a cell monolayer, which forms a physical barrier across which certain molecules may be transported, depending on the nature of the molecule. Alternatives to cell monolayers may of course be utilized to simulate absorption. Alternatives typically comprise a biological structure capable of active transport and include, but are not limited to, organs of the digestive tract obtained from lab animals and reconstituted organs or membranes created *in vitro* from cells seeded in an artificial matrix. Simulated absorption includes any process which simulates aspects of physiological absorption of components of the natural composition, whether or not modified by digestive processes, from the digestive tract to the body fluids and tissues. Simulated absorption may be performed for a preparation over a wide range of time periods, and depends on the nature of the preparation, the biological activity being measured, and the membrane across which the bioactive component is absorbed. Simulated absorption can be for periods of time ranging from about 5 minutes to about 48 hours. More preferred embodiments involve time periods of time ranging from 15 minutes to about 4 hours. In the most preferred embodiment, simulated absorption is complete within about 2 hours.

Alternatively, animal models are used to simulate both digestion and absorption of natural compositions. Animals are fed a natural composition and bioavailability is ascertained by testing, for example, serum or blood for the presence or absence of a component or for a specified biological activity. Bioactivity is ascertained in an animal by monitoring physiological or behavioral changes.

There is virtually no limit to the types of *in vitro* assays for measuring activity of natural compositions which are useful within the current invention. The search for such assays may typically start, for example, with a literature search of clinical studies that have demonstrated a positive clinical effect for the natural product being evaluated. The literature may then also be searched for studies that describe the biological mechanism or mechanisms associated with the clinical benefit. Particularly useful studies are those that define or suggest a mechanism

of action for the specific composition of the natural product that was used in a successful clinical trial. The range of functionality for the natural product is then carefully evaluated for each mechanism of action. An appropriate range of functionality may be established using statistical models known in the art. Samples of the natural product may thereafter be selected and evaluated according to the range of functionality which has been established. One or more *in vitro* bioassays may be selected for use with the natural compositions and these bioassays may be selected based on one or more of the following criteria: relatedness of the bioassay to the clinical effect seen in humans; reproducibility; cost-effectiveness; and/or efficiency as a quality assurance tool.

Other methods for determining the presence of components within a natural composition include, but are not limited to determinations by physical, chemical or immunological assays or tests. While it is the biological activity that is of interest, it can be the case that a biological response to an active component is manifested over a long period of time, or that an active component accumulates to a useful level in a biological system after multiple doses of the preparation are administered. In these situations, alternative methods for determining active components are particularly useful. In the event that a particular component of a natural composition has been identified, bioavailability of that component following simulated digestion and/or absorption need not be determined by an assay related to its biological activity. Any convenient method may be used to determine its presence in a preparation provided by subjecting the natural composition to simulated digestion and/or simulated absorption. Testing for the presence of a component may be performed concurrently with or following the simulated digestion and/or absorption step. Concurrent testing means testing that overlaps a digestion or an absorption step to some degree, but need not occur simultaneously.

In another aspect of the invention, there is provided a natural product useful as a dietary supplement, comprising a natural composition obtained from a batch of the natural composition, wherein representative samples of the batch are subjected to a method for evaluating biofunctionality. Such natural products are distinguishable over prior art natural products in that, *inter alia*, the natural products of the present invention have greater bio-

functional or compositional consistency as compared with prior art natural products. Evidence of such greater consistency is established, for example, by collecting a number of separately packaged samples of the natural products of the invention and comparing the biofunctional consistency of this collection with a number of prior art samples so that a statistical inference can be made.

The invention is useful for determining biofunctionality of natural compositions and to ensure consistent biofunctionality of natural products, such as dietary supplements. This includes, for example, any unprocessed of processed plant material as well as various standardized extracts or combinations of extracts and whole herbal concentrates and is not limited to natural products useful as dietary supplements. The assurance of quality and consistency provided by methods of the invention is equally applicable to raw and processed natural compositions, for example those compositions which are raw material for production of dietary supplements. Thus, the invention includes natural products having substantial batch-to-batch consistency, where the consistency has been evaluated by any of the above methods.

For each natural composition, unique functional characteristics and assays to optimally measure those characteristics are determined. In a certification stage, the assays are repeated with clinically proven products to establish an allowable range of biofunctionality. Once the range of allowable biofunctionality has been established for a product, the assays are useful for testing individual batches. The goal of such testing is to establish that each batch consistently maintains desired characteristics, such as those established in the initial certification stage.

The analytical methods of the current invention are applicable to a wide variety of natural preparations. A particular feature of the present invention is that the assays for testing biological activity are selected on the basis of biological mechanisms which are likely to account for the biofunctionality of the natural preparations. Typically this might mean selecting only two or three mechanisms for which the biological activity is to be tested.

For some natural products and compositions, it will be desirable to ensure that the level of a component or biological activity is above a minimum value. For other natural products, it can be desirable to ensure that a component or biological activity is within a certain range. In some cases, certifiable products will be within about 50% of a standard composition or activity. More preferably, such products will be within 75% of a standard composition or activity. Most preferably, those products will be within 90% of a standard composition or activity.

Any product may be tested and certified with any assay, depending only upon the use for which the product is sold. For example, it will be useful to test an herbal product having more than one application according to different methods or standards depending on its intended use. Similarly, it may be desirable to test different products having the same intended use by the same method.

Another feature of the present invention is that the range of biological activity can be selected to be that which is known to produce a desired biofunctionality, such that clinical testing may be conducted much more rapidly and cost effectively. Prescreening of materials prior to use in clinical trials allows test boundaries to be established which result in reduced group sizes with a higher degree of response.

In an embodiment of the invention, biofunctionality of a natural composition comprising *Echinacea* or an extract thereof is assessed. Laboratory studies have shown that components of *Echinacea purpurea* possess immunostimulatory activity toward murine and human mononuclear cells (Stimpel *et al.*, 1984; Wagner *et al.*, 1988; Luettig *et al.*, 1989; Roesler *et al.*, 1991; Burger *et al.*, 1997; See *et al.*, 1997). *Echinacea*-activated macrophages displayed enhanced proliferation, cytokine production, phagocytic activity, and the capacity to kill tumor cells as well as bacterial and fungal pathogens (Stimpel *et al.*, 1984; Coeugniet *et al.*, 1987; Wagner *et al.*, 1988; Leuttig *et al.*, 1989). When a natural composition of the invention comprises *Echinacea* or an extract of *Echinacea*, the test of biological activity can comprise testing the preparation for immunostimulatory activity. Immunostimulatory activity means the capacity to stimulate an activity of an immune system cell. Such cells include B cells, T

cells, macrophages and the like. Such cells of one cell type may be activated by such stimulation, or it can be the case the stimulation of one cell type results in immune cell interactions which cause activation of other cell types. Activation includes, but is not limited to induction of cell proliferation, secretion of cytokines or other signaling molecules, and secretion of oxygen carrying molecules, such as nitric oxide or peroxide. In certain embodiments of the invention, immunostimulatory activity includes the capacity of the preparation to stimulate phagocytic cells such as macrophages. A preferred macrophage cell is RAW264.7 and a preferred measure of immunostimulatory activity is production of Tumor Necrosis Factor. Activities of these cell types can also activate other cell types.

According to the present invention, the activity or compositional consistency of an *Echinacea* composition is determined by first subjecting the natural composition to simulated digestion and/or simulated absorption, followed by biological activity testing of the resulting preparation to determine its effect on lymphocyte activity or phagocytosis. The amount of the *Echinacea* composition that can be assayed for biological activity by the method of Example 1 can vary significantly. For example, the amount of *Echinacea* that is subjected to simulated digestion can be from about 10 mg to about 5000 mg. More preferably, the amount is from 100 mg to 2500 mg, Most preferably, the amount is about 750 mg. In one embodiment of this aspect of the invention, immunostimulatory activity of an Echinacea composition, extract or preparation is determined by monitoring the production of a responsive cytokine. For example, macrophage activation is detected by assessing production of TNF-α (Leuttig *et al.*, 1989). The amount of Echinacea composition used to stimulate production of TNF-α can be from about 0.1 μg/ml to about 5 μg/ml. more preferably from 0.5 μg/ml to about 2.5 μg/ml and most preferably 1 μg/ml. Other responsive species include interleukin-1, interleukin-6, interleukin-8, nitric oxide, reactive oxygen species and arachadonic acid metabolites. Commercial kits for measuring such species are readily available. Macrophages can be monitored for phagocytosis or killing of bacteria. In addition, macrophage activation and cytokine production supports the development of other immune cell responses, such as natural killer (NK) cell activity and antibody-dependent cellular cytotoxicity (ADCC) (Delfino *et al.*, 1991; See *et al.*, 1997).

Alternatively, the effect of a preparation on immunostimulatory activity is determined by assessing cell proliferation and viability. Macrophage activation and cytokine production support the development and activation of other immune cell types such as NK cells and lymphocytes (See *et al.*, 1997; Delfino *et al.*, 1991). *Echinacea*-derived polysaccharides do not induce substantial proliferation of isolated lymphocytes (Luettig *et al.*, 1989) but lymphocyte proliferation and NK cell activation does occur *in vitro* from stimulation of peripheral blood mononuclear cells (macrophages, NK cells, and lymphocytes; PBMCs) *in vitro* (See *et al.*, 1997) and *in vivo* (Coeugniet *et al.*, 1987). In an embodiment of the invention, PBMCs are treated with *Echinacea* compositions which have been prepared by methods of the invention and viable cell number are assessed. Final concentrations of *Echinacea* preparations can be used in concentrations from about 0.1 μg/ml to about 5 μg/ml. more preferably from 0.5 μg/ml to about 2.5 μg/ml and most preferably 1 μg/ml. A preferred and easily performed method for assessing viable cell numbers consists of monitoring conversion of the metabolic dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Other metabolic dyes are well know and can also be used. Methods for assessing viability also include, but are not limited to, measuring uptake of labelled nucleotides or amino acids (e.g., ^{3}H-thymidine) and measuring ATP levels by bioluminescence (e.g., by ViaLight kit, LumiTech Ltd., Nottingham, U.K.). Methods for assessing macrophage stimulation are not limited to detection of TNF-α and evaluation of cell proliferation. Other methods include, but are not limited to detection of synthesis of prostaglandins, other cytokines like IL-1β, IL-1α, nitric oxide, and oxygen radicals like superoxide and peroxide. It is well known in the art to measure cytokines and biological activities produced by cultured cells. For example, ELISA kits and cell proliferation assay kits to measure cytokines are readily available.

In another embodiment of the invention, biofunctionality of a natural composition comprising *Ginkgo biloba* or an extract thereof is tested. Recent studies have described the beneficial effects of *Ginkgo biloba* extracts (GBE) on patients with different types of dementias, mood changes, disturbances in cognitive functions associated with aging and senility, poor concentration and memory, confusion, depression and anxiety (Warot *et al.*, 1991; Hofferberth, 1989). *In vivo* laboratory studies have also shown that aging animals

administered *Ginkgo biloba* extracts have fewer degenerative structural changes in the hippocampus compared to control mice (Barkats *et al.*, 1994). GBE typically comprises four different phytochemicals which include flavone glycosides, terpenoids, proanthocyanidins, and organic acids. Flavonoids have been shown to scavenge free radicals and act as antioxidants. Free radical oxidation reactions are believed to be part of the aging process and are presumed to be major contributors to mental decline. Oxidative stress is implemented in the pathogenesis of several neurodegenerative disorders. *Ginkgo* flavonoids have been shown to protect cells from lipid peroxidation and cell death (Joyeux *et al.*, 1995; Robak *et al.*, 1988), protect neurons from necrosis and apoptosis induced by oxidative stress (Oyama *et al.*, 1996; Ni *et al.*, 1996), and reduce oxidative metabolism in both resting and Ca^{2+} loaded neurons (Oyama *et al.*, 1994). In an embodiment of the invention, activity of a *Ginkgo biloba* composition is tested by first subjecting the natural composition to simulated digestion and/or simulated absorption, followed by biological activity testing of the resulting preparation to determine its ability to scavenge free radicals. The amount of Ginkgo biloba which is digested according to the method of example one can be from about 5 mg to about 1000 mg. More preferably, the amount is from 25 mg to 500 mg, Most preferably, the amount is about 180 mg. In one embodiment, free radical scavenging is observed by monitoring the decoloration of a free radical reagent. It will be apparent to one of average skill in the art that many other oxidants and methods of testing for their presence and inactivation can also be used to test compositions of *Ginkgo biloba* following simulated digestion and/or absorption. The ability to scavenge free radicals is tested by mixing *Ginkgo biloba* compositions in a solution containing free radicals. Preferred free radicals are detectable for example by absorbance, and scavenging is detected by a change in absorbance of the solution. The amount of *Ginkgo biloba* required can vary according to the free radical to be quenched. the Ginkgo concentration can be from about 1 μg/ml to about 500 μg/ml, more preferably 5 μg/ml to 200 μg/ml, most preferably from 10 μg/ml to 100 μg/ml.

In another aspect of the invention, biofunctionality of a natural composition comprising *Ginkgo biloba* or an extract thereof is tested for its ability to inhibit platelet activating factor (PAF). The terpenoid fraction of *Ginkgo* contains ginkgolides of which Ginkgolide B is a potent inhibitor of platelet activating factor (PAF) (Nunez *et al.*, 1986; Smith *et al.*, 1996).

PAF is also known to activate cells involved in inflammation (Braquet *et al.*, 1987), regulate cellular immune responses (Prescott *et al.*, 1990), alter blood rheology and vascular permeability (Kinn *et al.*, 1998). Neurologically, PAF is involved in shock responses and post ischemic events (Baker, 1995), reduces cerebral blood flow (Kinn *et al.*, 1998), modulates synaptic activity (Bazan *et al.*, 1995; Yue *et al.*, 1994), plays an important role in brain development and is implicated in certain CNS disorders (Hattori, 1994; Smith *et al.*, 1996). Ginkgolide B has been shown to inhibit most of these functions of PAF in experimental models. In an embodiment of the invention, inhibition of platelet activation is assessed by determining the ability of a composition of *Ginkgo biloba*, following simulated digestion and/or absorption, to inhibit platelet aggregation. Numerous other way to assess inhibition of an activity associated with a platelet interaction will be apparent to one of skill in the art and can be applied to the present invention. Platelet activation and inhibition thereof can be measured in an aggregometer. Methods for making platelet rich plasma are well known in the art. A mixture is provided containing platelets, an agonist (platelet activating factor) which induces platelet activation and a *Ginkgo biloba* composition. The agonist can be Racemic-PAF. A preferred agonist is L-PAF. The concentration of the agonist can be from about 0.001 μM to about 0.1 μM, more preferably from 0.003 μM to 0.05 μM and is most preferably about 0.006 μM. A range of concentration can be chosen for the *Ginkgo biloba* composition being tested. A standard Ginkgolide may be employed and inhibition of platelet activation compared to that of the *Ginkgo biloba* compositions and preparations. The concentration of Ginkgolide standard can be from about 0.05 μM to about 10 μM, and is more preferably from 0.25 μM to about 2.5 μM, and is most preferably 1.0 μM.

In yet another embodiment of the invention, the biological activity of *Ginkgo biloba* which protects neuronal cells from toxicity associated with certain substances and growth conditions is assayed. On the basis of recent progress in the understanding of the onset and progression of Alzheimer's disease and the believed beneficial effects of *Ginkgo biloba* on age related cognitive disorders, we developed assays which mimic individual steps of neurodegenerative processes and which can be used to assess biological activity of *Ginkgo biloba* compositions. Substances and conditions known to be toxic to neuronal cells include high levels of an

excitatory amino [redacted] (glutamate), and amyloid-β peptide ([redacted]ssens *et al.*, 1995; Behl *et al.*, 1994). Serum starvation also promotes apoptotic death of cultured neuronal cells. In an embodiment of the present invention which comprises a novel assay for neuroprotection, neuronal cells are cultured under known toxic conditions. Neuronal cells are subjected to the aforementioned conditions or combinations thereof in the presence and absence of *Ginkgo biloba*. The ability of compositions of *Ginkgo biloba* to reduce or prevent toxicity is determined by assessing cell viability. Insults against which *Ginkgo biloba* has demonstrated protection include the following: (1) induction of apoptosis by serum starvation; (2) glutamate induced excitotoxicity; (3) cytotoxicity of conditions mimicking ischemia; (4) cytotoxicity of unpolymerized amyloid-β peptide; (5) cytotoxicity of fibrillar amyloid-β peptide; (6) induction of apoptosis in the presence of high concentrations of glutamate; (7) induction of apoptosis in the presence of amyloid-β peptide; (8) cytotoxicity of conditions mimicking ischemia followed by excitotoxic insult; (9) cytotoxicity of amyloid-β peptide in the presence of Fe ions; (10) cytotoxicity of amyloid-β peptide in the presence of high concentrations of glutamate; (11) induction of apoptosis in the presence of amyloid-β peptide and high concentrations of glutamate. Other conditions and combinations thereof which are toxic to neuronal cells will be evident to those of average skill in the art and can be used to assess the biological activity of a *Ginkgo biloba* composition. In a neuroprotection assay of the invention, a mixture is provided containing neuronal cells, a cytotoxic substance in an amount sufficient to reduce viability and a *Ginkgo biloba* preparation. The cytotoxic substance can be glutamate which is present in a concentration from about 1 mM to about 50 mM, and is more preferably from 2.5 mM to about 20 mM, and is most preferably 10 mM. The cytotoxic substance can be amyloid-β peptide and be present from about 0.05 μM to about 10 μM, more preferably from 0.2 μM to about 1.0 μM, and most preferably 0.5 μM. Extracts and compositions are tested to determine the extent to which they inhibit cytotoxicity.

The invention is also useful for assessing the biofunctionality of a composition comprising St. John's Wort or an extract thereof. St. John's Wort has been demonstrated to be effective against mild to moderate depression (Chatterjee *et al.*, 1998a; Laakmann *et al.*, 1998) with

few side effects (Wurbach *et al.*, 1997; Linde *et al.*, 1996). Although the mode of action is not known, *in vitro* bioassays indicate that inhibition of serotonin re-uptake and dopamine re-uptake are the predominant specific activities (Muller *et al.*, 1997). Serotonin re-uptake inhibition is a well established mode of action of many synthetic anti-depressants.

Other modes of action are possible for St. John's Wort. Embodiments of the invention are not limited to assays for serotonin re-uptake inhibition, and can include, for example, assays for inhibition of monoamine oxidase (Bladt *et al.*, 1994; Thiede *et al.*, 1994) or inhibition of GABA binding to the GABA receptor (Baureithel *et al.*, 1997; Cott, 1997).

In an embodiment of the invention, a composition comprising St. John's Wort is subjected to simulated digestion and/or simulated absorption, and the resulting preparation is tested for serotonin re-uptake inhibition activity. The test of biological activity can comprise providing a mixture containing the St. John's Wort preparation, a synaptosomal preparation and a neurotransmitter selected from the group consisting of dopamine and serotonin, and detecting uptake of the neurotransmitter by the synaptosomal preparation. Synaptosomes can be prepared, for example, from brain tissue by homogenization in ice-cold 0.32M sucrose solution as described elsewhere herein. In preparing synaptosomes, brain tissue volume can be approximated by weighing and the amount of sucrose solution added can be about one volume to about 50 volumes, and is more preferably from about 5 volumes to 15 volumes and most preferably is about 9 volumes. The amount of synaptosome preparation use in the assay mixture can be from about 5 μl to about 1 ml, and is more preferably 20 μl to 250 μl and most preferably 50 μl. The neurotransmitter can be dopamine or serotonin, and can be present in the extract at a concentration from about 2 nM to about 500 nM, more preferably from 5 nM to 100 nM, and most preferably about 50 nM. The amount of the St. John's Wort composition that can be assayed for biological activity by the method of Example 1 can vary significantly. For example, the amount of St. Johns Wort that is subjected to simulated digestion can be from about 10 mg to about 5000 mg. More preferably, the amount is from 100 mg to 2500 mg, Most preferably, the amount is about 900 mg. A convenient way to measure uptake use a radiolabeled neurotransmitter. Examples of convenient labeled neurotransmitters ate ^{14}C-serotonin, ^{3}H-serotonin, ^{3}H-dopamine. The re-uptake measurement

period can be fro out 15 seconds to about 15 minutes, [redacted]s more preferably 1 to 10 minutes and is most preferably 5 minutes.

It is found herein that, after simulated digestion St. John's Wort preparations retain serotonin re-uptake inhibition activity, although at a lower level than in the starting material. Loss of activity appears to take place during gastric digestion rather than intestinal digestion. This highlights an important feature of the invention which is the ability to simulate bioavailability from different formulations of a natural product. For example, formulations which prevent dissolution of an a natural dietary supplement until it has passed through the gastric environment into the intestinal environment will significantly improve bioavailability of active components which are acid labile. Thus, in another embodiment of the invention, formulations of natural products can be tested for loss of biological activity by simulated digestion and absorption, and selected on the basis of preservation of such activity.

In another embodiment, the present invention is useful for assessing the activity of a composition comprising saw palmetto or an extract thereof. Saw palmetto as a dietary supplement most commonly consists of an extract derived from the fruit of the American Dwarf Palm Tree (*Serenoa repens*). In Europe, saw palmetto is a well accepted and clinically studied remedy for symptoms associated with benign prostate hyperplasia (BPH). While the exact etiology of BPH is not fully elucidated, the metabolism and action of androgenic steroids and conversion of testosterone to dihydrotestosterone (5HT) by 5α-reductase appears to be strongly associated with the development of BPH (Strauch *et al.*, 1994; Isaacs *et al.*, 1983). Additional factors that exacerbate BPH are inflammatory mediators such as prostaglandins and leukotrienes produced by infiltrating leukocytes as well as localized growth factors such as basic fibroblast growth factor (bFGF) (Robinnette, 1988; Theyer *et al.*, 1992).

In vitro activities described for saw palmetto to explain the clinical benefits reported in BPH patients include inhibition of 5α-reductase (Strauch *et al.*, 1994; Délos *et al.*, 1994), inhibition of androgen binding to androgen receptor and activation of androgen-responsive reporter genes (Sultan *et al.*, 1984; Ravenna *et al.*, 1996), antiestrogenic activity (Di Silverio

et al., 1992), blo[redacted]e of prolactin and fibroblast growth f[redacted] signal transduction (Vacher *et al.*, 1995; Paubert-Braquet *et al.*, 1998), and inhibition of enzymes (cyclooxygenase and lipoxygenase) that produce inflammatory prostaglandins and leukotrienes (Paubert-Braquet *et al.*, 1997). *In vivo*, saw palmetto has been shown to block androgen-mediated prostate growth in castrated animals and pharmacokinetic labeling experiments have shown that major components of saw palmetto, (e.g., linoleic acid, oleic acid) distribute to the prostate (Chevalier *et al.*, 1997; Paubert-Braquet *et al.*, 1996).

However, these studies have not yielded any conclusive data on the possible mechanism of action of saw palmetto *in vivo*. For example, Proscar dramatically reduces the levels of circulating 5HT and levels of prostate specific antigen (PSA), an androgen regulated gene product (Strauch *et al.* 1994; Rhodes *et al.* 1993). In some studies patients using saw palmetto consistently displayed no statistically significant decrease in the levels of these substances in serum (Casarosa *et al.* 1988; Braeckman, 1994). In contrast, recent studies have shown that administration of saw palmetto extracts does lower 5HT within the prostate of BPH patients, suggesting inhibition of 5α-reductase in the prostate as a mechanism of action of saw palmetto (Di Silverio *et al.* 1998).

In embodiments of the invention, compositions comprising saw palmetto and extracts thereof are assessed for their capacity to inhibit 5α-reductase. In a particular embodiment, biological activity is assessed by measuring inhibition of metabolism of one or more 5α-reductase substrates (e.g., testosterone and/or progesterone) in a mixture which comprises 5α-reductase from rat liver microsomes, NADPH and the 5α-reductase substrate(s) and the saw palmetto composition. The saw palmetto composition is present at a concentration between about 5 μg/ml and about 500 μg/ml, more preferably between 10 μg/ml and 100 μg/ml, and most preferably at about 50 μg/ml. When microsomes are prepared at equivalent volume as given in Example 5, the amount of the microsome preparation present in the assay can be from about 2 μl to about 200 μl, more preferably 5 μl to 50 μl, and most preferably 20 μl. 5α-reductase substrates are present at from about 5 μg/ml to about 500 μg/ml, and more preferably 20 μg/ml to 200 μg/ml. Most preferably, the testosterone concentration is

25 μg/ml and th gesterone concentration is 20 μg/ml. reaction is started by the addition of NADPH to a final concentration of about 20 μg/ml to about 2 mg/ml, more preferably 50 μg/ml to 1 mg/ml, and most preferably 490 μg/ml. The incubation is between about 30°C and about 40°C, more preferably between 35°C and 39°C, and most preferably at about 37°C for a period of time from about 5 minutes to about 2 hours, more preferably 30 minutes to 90 minutes and most preferably for about 1 hour.

A likely mode of action, as discussed herein, is inhibition by SPE of the transformation of androgen receptor to the DNA binding form. It has been discovered that SPE inhibits the transformation of aryl hydrocarbon receptor to the DNA binding form. Based on the present discovery that SPE inhibits transformation of aryl hydrocarbon receptor and a critical review of the literature, it is believed herein that SPE is a noncompetitive, non-specific inhibitor of HSP90-bound hormone receptor transformation. By use of the term "non-specific," it is meant that SPE has the capacity to inhibit transformation to DNA binding form of aryl hydrocarbon receptor, androgen receptor, and perhaps other receptor proteins which can undergo a like transformation. While aryl hydrocarbon receptor is not part of the androgen or estrogen receptor family, nor directly involved in hormone signal transduction, all these receptors use similar mechanisms to maintain unliganded competence and for activation to the liganded DNA-binding form, and all have corresponding defined DNA response elements to which they bind. An anti-estrogen effect has been shown for SPE which prevents the appearance of nuclear estrogen receptor (Di Silverio *et al.*, 1992; Bombardelli and Morazzoni, 1997). IC_{50} for the tested SPE is found to be 20 μg/ml. This is in the same range found for enzyme inhibition activity for SPE.

In such an assay, androgen response element DNA is diluted in cytosol or nuclear extract which is the source of unliganded androgen receptor bound to HSP90, to a concentration between about 0.01 nM and about 1000 nM, more preferably between 0.1 nM and 10 nM, most preferably about 1.0 nM. Androgen response element may be biotinylated depending upon the detection method employed. Microsomes are prepared as above. The amount used in an assay can vary between about 30 μl and about 3000 μl, more preferably between 100 μl and 1000 μl, and is most preferably about 500 μl. Testosterone is present between about

0.5 μg/ml and 2[redacted]g/ml, more preferably between 1 μg/[redacted]d 100 μg/ml, and most preferably is about 50 μg/ml. Saw palmetto material and NADPH are then added to the mixture. Concentrations for each may be about 1.0 μg/ml to about 1000 μg/ml, more preferably 10.0 μg/ml to 200 μg/ml, and most preferably about 100 μg/ml. Similar conditions may be employed for assays based on aryl-hydrocarbon receptor and response element. Methods for detecting the formation of complexes such as those between androgen response element DNA and androgen receptor are well known in the art. For example, they can be immunologically based, relying on binding of an element to a solid support.

Thus, an embodiment of the invention which tests for biological activity of a saw palmetto composition or extract comprises providing a mixture containing the saw palmetto preparation, 5α-reductase, cofactor NADPH, testosterone, unliganded androgen receptor complexed with HSP90 and androgen response element DNA, and detecting the formation of an androgen receptor-androgen response element complex. Alternatively, the test comprises providing a mixture containing the saw palmetto preparation, unliganded aryl hydrocarbon receptor complexed with HSP90 and aryl hydrocarbon response element DNA, and detecting the formation of an aryl hydrocarbon receptor-aryl hydrocarbon response element DNA complex.

In another aspect of the invention, there is provided a novel assay capable of monitoring anti-androgenic inhibition by SPE of the transformation of androgen receptor. This assay provides capability to simultaneously measure the contribution to anti-androgenic activity from inhibition of 5HT formation by 5α-reductase.

A particularly useful aspect of this novel assays is that it provides a measurement of biological activity of saw palmetto extracts and commercial products as well as saw palmetto-containing compositions prepared by simulated digestion and absorption methods of the current invention.

A frequent occurrence with BPH is a hormone-induced chronic inflammation that results from infiltration of inflammatory macrophages and neutrophils into the prostate (Theyer *et*

al., 1992). Production of prostaglandins such as prostaglandin E_2 (PGE2) and leukotrienes (leukotriene B4, LTB4) are known inflammatory mediators produced by these cells (Robinette, 1988; Theyer *et al.*, 1992). Previous investigations have reported that *Serenoa repens* extracts inhibit the cyclooxygenase (COX) enzymes *in vitro* and that components of *Serenoa repens* extracts that concentrate in the prostate also inhibit these enzymes (Paubert-Braquet *et al.*, 1997; Chaudry *et al.*, 1994). In addition, one study has indicated that decreases in urinary PGE_2 concentrations are correlated with urinary flow improvement (Rolland *et al.*, 1981). Thus, embodiments of the invention include those which assess the anti-inflammatory activity of a saw palmetto composition or extract. Previous studies implicating infiltration of inflammatory immune cells suggest the central role of secreted mediators of inflammation (e.g., cyclooxygenase and macrophage derived cytokines) in diseases which are ameliorated by saw palmetto. Furthermore, pharmacokinetic data indicating *in vivo* bioavailability at the target site exists and supports an anti-inflammatory mechanism of action for saw palmetto (Chaudry *et al.*, 1994; Plosker *et al.*, 1996). As discussed above, macrophage activation can be measured in a variety of ways. In a preferred embodiment, levels of biological activity of a saw palmetto composition or extract is assessed by measuring inhibition of production of prostaglandin E_2 (PGE_2).

In an embodiment of the invention, compositions and extracts of *Panax ginseng* are evaluated for biological activity and compositional consistency. *Panax ginseng* has been used in traditional Chinese medicine for approximately 2000 years to enhance stamina and improve responses to fatigue and stress. Although mechanisms of action remain unclear, effects have been observed which involve the central nervous system (memory, learning and behavior; D'Angelo *et al.*, 1986), neuroendocrine function (Hiai *et al.*, 1979), immune function (Scaglione *et al.*, 1996), antioxidant actions (Kim *et al.*, 1996), carbohydrate and lipid metabolism (Wink *et al.*, 1993), and the cardiovascular system (Rimar *et al.*, 1996; Fukuda *et al.*, 1995).

Ginseng is thought to act on the hypothalamus, causing release of corticotropin, which in turn acts on the pituitary to release ACTH, which acts on the adrenals to release corticosteroids. The release of corticotrosteroids and adrenocorticotropic hormone (ACTH) act on the brain

and body to provoke stress adaptation (Fulder, 1980; Fulder, 1981). Ginseng exhibits a potentially synergistic effect by also binding and activating glucocorticoid receptors, mineralcorticoid receptors and progesterone receptor (Pearce *et al.* 1982; Lee *et al.* 1997).

Activities attributed to ginseng are believed to be due to ginsenosides, which have been shown to bind and activate glucocorticoid receptors as well as bind to mineralcorticoid and progesterone receptors. The most commonly studied activities of glucocorticoids involve the modulation of nitric oxide synthase isoenzymes, especially the inhibition of inducible nitric oxide synthase (iNOS) (Di Rosa *et al.* 1990; Park *et al.* 1996). Glucocorticoid receptor-mediated inhibition of iNOS expression under stress/inflammatory conditions has beneficial effects on blood flow, maintenance of cardiac muscle contractility, and beneficial immunological activities, permitting expansion of T and B lymphocytes and up-regulation of cytokine receptors on immune cells (Radomski *et al.* 1990; Hawrylowicz *et al.* 1994).

Embodiments of the invention enable the evaluation of *ginseng* extracts and compositions for biological activities which stimulate corticosteroid induction and inhibit nitric oxide production. In a preferred embodiment, a ginseng extract is administered to an animal and levels of corticosterone are measured in blood plasma. In another preferred embodiment, ginseng extracts are tested for their ability to inhibit nitric oxide production in cultured macrophage cells activated with IFN-γ

The examples which follow describe the invention in detail with respect to showing how certain specific representative embodiments thereof can be made, the materials, apparatus and process steps being understood as examples that are intended to be illustrative only. In particular, the invention is not intended to be limited to the methods, materials, conditions, process parameters, apparatus and the like specifically recited herein.

Throughout this application, various publications, patents, and patent applications have been referred to. The teachings and disclosures of these publications, patents, and patent applications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

It is to be understood and expected that variations in the principles of invention herein disclosed may be made by one skilled in the art and it is intended that such modifications are to be included within the scope of the present invention.

EXAMPLES OF THE INVENTION

Example 1

Simulated Digestion and Absorption of Natural Compositions for Assessment of Biofunctionality. Determination of the benefit provided by a natural composition after consumption is improved by testing the material in bioassay(s) following a simulated digestion and/or absorption process. Simulation of these physiological processes adds stringency to the assessment of biofunctionality, the result being a better indication of potential *in vivo* activity. Because herbs and natural extracts are complex materials, digestion enhances or reduces absorption and subsequent biological activity.

Simulated digestion. A final formulation composition (tablet, capsule, soft gelatin capsule, etc.) was added to 15 ml of simulated gastric fluid prepared following the method of USP volume 23. The gastric fluid had a pH of approximately 1.2 and contained the gastric enzyme pepsin, thus simulating the digestive environment of the stomach. The contents were then shaken at 250 RPM at 37°C for 2 hours for dissolution of the test material.

Following this incubation, the pH of the resulting mixture was then adjusted to approximately 7.4 by the addition of 0.5 ml of a 2.2N solution of sodium hydroxide and the addition of an equal volume (15.5 ml) of 2X concentrate of simulated intestinal fluid containing 2X pancreatin. The flask was shaken at 250 RPM at 37°C for 2 hours. This resulting solution was an approximation of physiologic intestinal fluid containing pancreatic enzymes that the test material would be exposed to upon oral consumption. The material was then flash frozen and stored at -80°C for further testing.

Simulated absorption and estimation of bioavailability. Apart from the effects of a digestive environment as may be simulated by the procedure above, the biofunctionality of a

composition depends on its ability to exit the digestive tract and enter the circulation to reach the site(s) of action. A good *in vitro* method for assessment of absorption and bioavailability has been demonstrated using a colon carcinoma cell line, Caco-2, which when plated at high densities differentiates into an intestinal epithelial-like monolayer (S. Yee, 1997). A layer of differentiated Caco-2 cells on plates that allow the flow of material from apical to basolateral and vice versa, is used to assay absorption and bioavailability of a test material. This model system is well characterized and has been shown to correlate with *in vivo* bioavailability studies performed in animals and humans (Yee, 1997; Taylor *et al.*, 1997).

Caco-2 cells purchased from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in T-75 culture flasks with Dulbecco's Modified Eagle's Media (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA) and 100 units/ml penicillin - streptomycin solution (Sigma, St. Louis, MO). For bioavailability assessment, cells were plated onto Transwell membrane filters (Corning-Costar, Cambridge, MA) and once confluent, were maintained for about 21 days for differentiation into an intestinal epithelial monolayer.

A convenient alternative to Caco-2 cells is the BIOCOAT® Intestinal Epithelium Environment system (Becton Dickenson, Franklin Lakes, NJ. The kit can provide a confluent layer of cells in about 3 days.

Test material may either be dissolved in DMSO or 50% ethanol, or may consist of an aliquot from a simulated digestion protocol (see above). Samples from the digestion protocol were diluted or incubated at 50°C to heat inactivate pancreatic enzymes. This step is used to remove pancreatic enzymes which may be toxic to cells and thus could ruin the integrity of the Caco-2 monolayer. The test sample was then added to the apical side of the cells and then incubated at 37°C. Incubation temperature can be lower. The BIOCOAT system allows assays to be performed at room temperature and can with incubation times from about 10 minutes to about 2 hours or longer. Aliqouts for biofunctional testing were taken from the apical and basolateral compartments at 30, 60 and 90 minutes following administration of the test material. Where bio-functional testing is performed using cell lines capable of growing

in the same culture media as the Caco-2 monolayer, the procedure can performed using cell culture wells containing the target test cell line on the basolateral side of the monolayer. The transwells and the supported Caco-2 cell monolayer are removed once absorption is complete.

Validation of simulated digestion protocol. In order to validate the simulated digestion procedure outlined above, two types of analysis were performed. In the first analysis, procedural consistency of simulated digestion was determined. Table 1 depicts pH after completion of simulated digestion of various botanical materials for experiments performed on three different days. In the second analysis (Table 2), biological activity was assessed for two different botanicals using material from at least three different digestions of the same lot of starting material. Activity was assessed using the *Echinacea* Macrophage Activation Assay (Example 2) or the *Ginkgo biloba* free radical scavenging assay (Example 3). The data shown in the Tables 1 and 2 show a relative standard deviation (CV), a description of variability, of less than 10% for simulated digestion and functional biological activity.

Table 1 - Consistency of Simulated Digestion

Product/Sample	pH Day -1	pH Day - 2	pH Day - 3
St. Johns Wort	6.76	6.65	6.83
Echinacea	6.85	6.66	6.88
Ginkgo biloba	6.85	6.75	6.88
Panax ginseng	7.02	6.80	6.98
Saw Palmetto	6.98	6.80	7.03

Table 2 - Consistency of Biological Activity		
Sample	**Response**	**CV**
Echinacea (digest 1)	1027 pg/ml TNF-α	9.2%
Echinacea (digest 2)	1165 pg/ml TNF-α	
Echinacea (digest 3)	977 pg/ml TNF-α	
Ginkgo biloba (digest 1)	EC_{50} = 44.60 μg/ml	7.7%
Ginkgo biloba (digest 2)	EC_{50} = 43.21 μg/ml	
Ginkgo biloba (digest 3)	EC_{50} = 50.01 μg/ml	
Ginkgo biloba (digest 4)	EC_{50} = 42.20 μg/ml	

Example 2

***In Vitro* Assay For Biological Activity of *Echinacea* Preparations.** *Echinacea* extracts and compositions prepared using the simulated digestion and absorption models described above were evaluated *in vitro* based on their ability to stimulate TNF-α production by macrophages and to stimulate cell proliferation of PBMCs.

Assay for Macrophage Production of TNF-α. RAW264.7 macrophage cells were plated at a density of $1x10^6$ cells/ml in 24- or 96-well dishes and incubated for 24 hours. Although the macrophage line has been found to produce small amounts of TNF-α upon plating, the amount of TNF-α in the culture medium decreases during the 24-hour incubation period. Echinacea compositions at various concentrations were added to RAW264.7 cells and TNFα concentration were determined. Echinacea compositions were dissolved standards, products and preparation prepared by simulated digestion or simulated digestion and simulated absorption. TNF-α levels were determined by ELISA using a mouse TNF-α ELISA kit (Endogen, Woburn, MA) and quantification was by comparison to a recombinant TNF-α standard.

Yield comparison. One important advantage of simulated digestion is simulation of the effects of ingestion on a natural composition. A second advantage is preservation of

biological activity of relevant components. Contents of capsules containing a powdered *Echinacea* product were prepared by overnight extraction with common solvents (dimethylsulfoxide or 50% ethanol) and by simulated digestion. Fig. 1 shows a comparison of TNF-α production by macrophages which was induced by a placebo and by the various preparations of the powdered *Echinacea* product. The *Echinacea* product prepared by simulated digestion retained a significant amount of TNF-α stimulating activity, whereas the products prepared by either of the common extraction methods demonstrated no activity above that of the placebo. Furthermore, as shown in Fig. 2, the macrophage activating capacity was dose dependent, indicating specificity.

Comparison of *Echinacea* raw materials and products. A variety of *Echinacea* raw materials and products were tested for macrophage activating activity after simulated digestion. As shown in Fig. 3, when compared to a reference material and a placebo control, only two of seven raw material batches demonstrated a significant capacity to induce macrophage TNF-α production. A comparison of samples of available *Echinacea* products is depicted in Fig. 4. Of eleven tested products, only three demonstrated a capacity to activate macrophages which was comparable to the reference.

Bioavailability. Reference product and placebo that had been subjected to simulated digestion were placed on the apical side of a Caco-2 cell monolayer. After two hours, samples were removed from the apical side and the basal side of the monolayer, diluted 10-fold and assayed for functional TNF-α activity. Fig. 5 shows the activity determined before and after simulated absorption for samples of the reference product and the placebo. The bars represent the mean ± standard deviation for three replicate assay wells.

To assess permeability of the Caco-2 monolayer, TNF-α amounts in the apical media at t=0 and in the basal media at t=2 hrs. were determined. Any effect of cytokine production by the Caco-2 cells was removed by subtracting placebo values from the values obtained for the reference product. 38,945 TNF units had been added to the apical side of the monolayer. Following the two hour incubation, 30,720 TNF units were measured on the basal side. Apparent permeability (P_{app}) was then assessed by the calculation of Yee (1997) as follows:

$P_{app} = (F*V_D) / (SA*M_D)$ where F = Units of activity / second (4.27), V_D = apical volume (0.5 ml), SA = membrane surface area (1.13 cm^2) and M_D = total Units in apical volume. Based on other studies, the calculated value, $P_{app} = 48 \times 10^{-6}$, corresponds to a predictive absorption for this material of about 70% or higher (Yee, 1997).

Certification of product. Five lots of an *Echinacea* product were tested for lot-to-lot consistency by simulated digestion. Four lots depicted in Fig. 6 induced TNF-α production in macrophages within a comparable range. The fifth lot induced significantly less activity. For assay certification, such comparative assays are combined with other studies to establish a desired range of biofunctionality within which certifiable product batches must fall. Thus certified, the assay is used to test batch samples to ensure quality. Only product which falls within the proper range of biofunctionality is certified.

Induction of cell proliferation. Peripheral blood mononuclear cells (PBMCs, Clonetics, San Diego CA) were treated with an *Echinacea* sample prepared by simulated gastric and intestinal digestion. A vial of cells (about 5×10^7 cells) was thawed and suspended in Lymphocyte Growth Medium (LGM-3, Clonetics) prewarmed to 37°C. One hundred μl of cell suspension was dispensed into each well of a 96-well microtiter tissue culture plate. The test samples, digested placebo capsules, *Echinacea* reference material, and Concanavalin A (Con A, positive control), were prepared to 10X concentrations in LGM-3 media and 10 μl of respective test material was added to the wells. The cells were incubated for approximately 72 hours and evaluated for proliferation/viability via conversion of the metabolic dye, MTT, using a Cell Titer™ Proliferation Assay Kit (Promega, Madison, WI). 1 ml of supplied MTT dye solution was added to 5 ml of LGM-3 solution and 50 μl of MTT/LGM-3 media was added to each well. After incubation at 37°C for 2 hours, 100 μl of stop/solubilization solution was added and the optical density at 570 nm was determined using a BioTEK Instruments ELISA plate reader. Table 3 shows the dose response relationship between *Echinacea* concentration and cell proliferation. Table 4 shows the dose response relationship between the positive control compound, Con A, and cell proliferation. Values for each concentration of *Echinacea* or Con A represent means of 8 wells. Statistical significance (P) was calculated using the one-tailed student's T-test versus placebo capsule response. Cell

proliferation peaked at a concentration of digested *Echinacea* which was about 1 μg/ml. Con A also produced a dose-dependent response and 1 μg/ml produced a high level of stimulation, and was thus chosen as a dose for following experiments.

Table 3 - Dose Response of *Echinacea* Reference Material and PBMC Proliferation

Echinacea Concentration (μg/ml)	Mean OD_{570}	Standard Deviation	%CV	P value
5	0.590	0.022	3.73	<0.01
1	0.668	0.036	5.40	<0.01
0.5	0.597	0.086	14.37	<0.01
0.1	0.560	0.099	17.74	<0.01
Placebo	0.425	0.050	11.72	
Concanavalin A (1 μg/ml)	0.790	0.035	4.39	<0.01

Table 4 - Dose Response of Concanavalin A and PBMC Proliferation

Concanavalin A Concentration (μg/ml)	Mean OD_{570}	Standard Deviation	%CV	P value
10	0.777	0.024	3.05	<0.01
5	0.714	0.018	2.58	<0.01
1	0.716	0.013	1.88	<0.01
0.5	0.463	0.027	5.9	<0.01
Placebo (1 μg/ml)	0.415	0.029	7.01	-

Table 5 shows the interassay variability and lot-to-lot reproducibility of the *Echinacea* standard for the PBMC proliferation assay using cells from a single donor. Table 6 demonstrates a significant proliferative response from PBMCs of three different donors. For each set of assays, eight replicate wells were assayed on three or more days using 1 μg/ml

digested *Echinacea* or Con A. All *Echinacea* lots and Con A were statistically different than placebo ($P<0.01$).

Table 5 - Reproducibility of PBMC Proliferative Response (OD_{570} Measurement)

Sample	Day 1	Day 2	Day 3	Day 4	3/4-Day Mean
Placebo	0.479	0.425	0.458	0.469	0.458
***Echinacea* Standard Lot #1**	0.579	0.654	0.665	0.664	0.641
***Echinacea* Standard Lot #2**	-	0.631	0.669	0.665	0.652
***Echinacea* Standard Lot #3**	0.542	0.629	0.692	0.641	0.626
***Echinacea* Standard Lot #4**	0.594	0.649	0.689	0.650	0.643
Con A	0.784	0.790	0.762	0.722	0.765

Table 6 - Proliferative Response of PBMCs from Different Donors (OD_{570} Measurement)				
Sample	**PBMC Donor #1**	**PBMC Donor #1**	**PBMC Donor #1**	**Intra-Donor Mean**
Placebo	0.407	0.295	0.361	0.355
***Echinacea* Standard Lot #1**	0.613	0.623	0.527	0.588
***Echinacea* Standard Lot #2**	0.572	0.591	0.553	0.587
***Echinacea* Standard Lot #3**	0.585	0.577	0.473	0.545
***Echinacea* Standard Lot #4**	0.606	0.612	0.497	0.572
Con A	0.761	0.904	0.735	0.800

Example 3

***In Vitro* Assays for Biological Activity of *Ginkgo biloba* Preparations.** Assays developed for *Ginkgo biloba* assessed the direct antioxidant activity of the herbal product, the ability to inhibit binding of platelet activating factor to its receptor and the ability of *Ginkgo biloba* to block neuronal cell death following either a mitochondrial insult or exposure to amyloid-β peptide.

***In vitro* Assay to Detect Free Radical Scavenging Activity.** 167 mg of diphenyl picryl hydrazyl (DPPH, Sigma, St. Louis, MO) was dissolved into 500 ml ethanol and stored in aliquots at -80°C. Assay buffer solution (BS) was prepared by mixing 20 ml of 1M Tris-HCl (pH 7.4) into 880 ml of 50% ethanol/water solution. Assays were performed in 96-well microtiter plates by first diluting the sample to be assayed into BS buffer to the top assay concentration, followed by two-fold serial dilutions of the sample in BS. 100 μl of DPPH solution was then added to all wells and the plate incubated at room temperature for 30 minutes. Optical density was measured at 540 nm.

Table 7 shows the mean percentage of scavenged free radicals for different concentrations of *Ginkgo biloba* compositions. The compositions were a standardized undigested Ginkgo extract, the same standardized extract subjected to simulated digestion and three different *Ginkgo biloba* products also subjected to simulated digestion. The results indicated that the free radical scavenging activity of *Ginkgo biloba* was affected by digestion. In addition, based on calculated EC_{50} values, it was evident that three different brands of *Ginkgo biloba* products which incorporated "equivalent" standardized extracts (24% flavonoids, 6% terpenoids) of *Ginkgo biloba* had significantly varying antioxidant potency.

Table 7 - Scavenged Free Radicals (%)

Extract Concentration (μg/ml)	Standardized Ginkgo Extract (DMSO)	Standardized Ginkgo Extract (Digest)	Product A (Digest)	Product B (Digest)	Product C (Digest)
200	100	100	100	100	100
100	100	100	98.1	100	79.7
50	97.9	77.3	59.5	79.2	45.7
25	58.2	43	34.2	45.7	27.4
12.5	33.4	24.6	19.4	24.0	14.5
6.25	18.1	13.0	11.2	13.7	7.9
3.12	9.0	6.2	5.7	5.3	4.5
EC_{50} (μg/ml)	**12.9**	**25.2**	**40.2**	**22.72**	**62.8**

***In Vitro* Assay for Inhibition of Platelet Activating Factor.** Blood was collected from the ear artery of fasted rabbits which had been free of medications and vaccinations for at least two weeks. Venipuncture was performed using a butterfly catheter (21 gauge x 3/4 inch needle; 3 ½ inch tubing). 1 ml of blood was withdrawn and discarded. 30 ml of whole blood was then withdrawn directly into a syringe containing 3 ml of 3.2% sodium citrate. The blood was gently mixed by inverting the syringe several times. Blood samples exhibiting

signs of hemolysis or fibrin formation were discarded. Platelet rich plasma (PRP) exhibiting signs of platelet activation (shape change), defined as a failure to exhibit "swirling" when held up to a light and gently mixed, was also discarded.

Preparation of PRP. The 30 ml anticoagulated blood was transferred to two 15 ml sterile plastic centrifuge tubes and PRP was isolated within 30 minutes by three successive centrifugation steps at 650 x g room temperature, and at 2-3 minute intervals. The PRP obtained after each step was transferred to a 15 ml sterile plastic tube, capped and allowed to stand for 30 minutes at room temperature prior to analysis of platelet function. The PRP was evaluated for evidence of activation (see above) and discarded if activated. Platelet poor plasma (PPP) was obtained by centrifugation of the remaining packed cell fraction at room temperature for 10 minutes at 2000 x g. PPP was transferred to a second sterile tube.

A 10 μl aliquot was removed from the PRP for platelet counting. The aliquot was diluted in 20 ml of isotonic cell counting solution and counted. Autologous platelet poor plasma (PPP) was used to adjust platelet number in the PRP to 300,000/μl.

Measurement of platelet aggregation. Optical platelet aggregometry was performed using a Chrono-Log platelet aggregometer at 37°C with stirring at 850 rpm. For each test, 450 μl PRP was added to the aggregation cuvette with a Teflon® stir bar, and the cuvette was placed in the measuring cell of the aggregometer. A cuvette containing 450 μl of autologous PPP, a stir bar, and 50 μl of 0.15 M NaCl (prewarmed to 37°C) was placed in the reference cell position of the aggregometer. The baseline was set using the set baseline mode. The maximal pen deflection (distance in mm) from the baseline for PPP relative to PRP is recorded on the chart. The PRP was prewarmed for 1 minute at 37°C with stirring followed by addition of 5 μl of inhibitor or extract in DMSO or other vehicle using a Hamilton micro syringe and allowed to react with platelets for 1 minute followed by addition of 5 μl of platelet agonist. The aggregation response profile was recorded for 3 minutes after agonist or control additions. The preferred agonist is L-alpha-phosphatidylcholine,beta-acetyl-gamma-O-hexadecyl (L-PAF, Sigma) which has

been found to have significantly higher activity than DL-alpha-phosphatidylcholine,beta-acetyl-gamma-O-hexadecyl (racemic PAF).

Platelet aggregation was determined as follows. Light transmission of the PRP solution after addition of a saturating level of PAF represented 100% platelet aggregation. Light transmission of a control solution of PPP after the addition of vehicle (e.g., DMSO) represented 0% platelet aggregation. Light transmission values were corrected for the effects of other components of the solution by performing parallel measurements of light transmission of a similarly treated PPP solution. To determine a linear range for the assay, test reactions were performed which contained different concentrations of L-PAF. To measure inhibition of *Gingko biloba* extracts, a concentration of L-PAF was chosen which was in a linear range of the assay. Platelet aggregation was determined according to the following equation: % Platelet aggregation = 100 x T_o / T_{max} where T_o is the increase of light transmission for a test reaction. T_{max} is the increase in light transmission for a control reaction which contains no *Ginkgo biloba* extract.

It was found that at low doses of L-PAF (< 0.1 μM), there was a linear relationship between L-PAF concentration and platelet aggregation. Maximal platelet aggregation was induced by concentrations above 0.1 μM. Table 8 shows that the linear dose-response and EC_{50} value obtained for L-PAF using different platelet preparations was highly reproducible.

To validate the assay method, results were obtained with Ginkgolide B and undigested *Ginkgo biloba* reference materials. Table 9 shows a dose-response analysis of the inhibition of L-PAF induced platelet aggregation by Ginkgolide B. Dose response curves were established at four different PAF concentrations representing different levels of platelet activation (% of maximal response). As expected for a competitive inhibitor of PAF-induced aggregation, the IC_{50} for Ginkgolide B was dependent on the extent of platelet activation. The data obtained for the linear regression coefficients showed that there was a linear response (based upon correlation coefficient, r) of platelet aggregation inhibition at and above 0.02 μM PAF. Therefore, this PAF concentration (0.02 μM) was used in further experiments to obtain the highest sensitivity with a linear response.

Table 8 - Dose-Response Relationship for Different Platelet Preparations			
Experiment #	**Rabbit ID #**	**L-PAF Platelet Activation (EC_{50})**	**Correlation Coefficient (r)**
1	7037	0.008	0.95
2	7037	0.006	>0.98
3	7037	0.006	>0.98
4	8052	0.006	>0.98
5	7261	0.006	>0.98
6	7036	0.006	>0.98

Table 9 - Inhibition of Platelet Aggregation by Ginkgolide B			
L-PAF Concentration (μM)	**Percent of Maximal Platelet Aggregation**	**Calculated IC_{50} (μM) for Ginkgolide B**	**Ginkgolide B Dose-Response Correlation Coefficient (r) of Linearity**
0.01	63%	0.2	0.90
0.02	87%	0.65	>0.99
0.05	90%	3.3	>0.99
0.075	96%	6.4	>0.99

Table 10 shows the reproducibility of the IC_{50} determination for Ginkgolide B, performed on three different days, for the selected 0.02 μM L-PAF concentration. r values were close to a perfect fit (r=1) for the determination of IC_{50} on different days. This demonstrates that inhibitor activity can be determined with high accuracy for the same platelet preparation.

Table 10 - Inhibiti n of Platelet Aggregation by Ginkgolide B (0.02 μM L-PAF)			
Experiment #	**% Maximal Platelet Aggregation**	**Calculated IC_{50} IC_{50} (μM?) for Ginkgolide B**	**Correlation C efficient (r) of Dose-Response**
1	87%	0.65	>0.99
2	89%	1.0	>0.99
3	89%	1.1	>0.99
	Mean Standard Deviation CV	**0.92 0.24 26%**	

Although the same PAF concentration does not necessarily result in the same level of platelet aggregation when different platelet preparations are used, the precision and reliability that is obtained is improved when measurements are made with reference to the activity of a *Ginkgo biloba* standard. IC_{50} values for *Ginkgo biloba* reference material and Ginkgolide B standard were determined for three independent platelet preparations at the selected PAF concentration. A linear relationship between *Ginkgo biloba* concentration and platelet aggregation inhibition is found in the tested range (r>0.99; data not shown). Moreover the activity of *Ginkgo biloba* relative to Ginkgolide B (Ginkgolide B IC_{50}/*Ginkgo biloba* IC_{50}) is independent of platelet preparations at the selected PAF concentration. Therefore, relative activity (*Ginko biloba* test material IC_{50}/*Ginkgo biloba* standard IC_{50}) can used to compare functional inhibitory activity on platelet aggregation for *Ginkgo biloba* preparations. Table 12 shows reproducibility achievable among different preparations of one lot and among different lots of the reference material.

Product certification. The minimum activity required for product certification depends on the accuracy and precision of the assay used to determine activity, reproducibility of data from the different preparations of the same reference material and variability between different lots of reference materials. The standard deviation shown by the assay in determining relative activity for the same reference material is low (2.7%, Table 11). As shown in Table 12 variability due to sample preparation within the same lot and variability among lots is also low. These considerations allow the selection of an activity level which

must be met for a product to achieve certification. Once validated, the assay is used to test *Ginkgo biloba* compositions and extracts which have been prepared by simulated digestion and/or absorption.

Table 11 - Inhibition of Platelet Aggregation by *Ginkgo biloba* Reference Material Relative to Ginkgolide B Standard

Experiment #	Percent of Activity of Ginkgolide B Standard
1	2.2
2	2.1
3	2.1
Mean **Standard Deviation** **CV**	**2.1** **0.06** **2.7%**

Table 12 - Inhibition of Platelet Aggregation by *Ginkgo biloba* Reference Materials Relative to Ginkgolide B Standard

Ginkgo biloba Preparation	Percent of Activity of Ginkgolide B Standard
Lot #1, sample preparation 1	2.2
Lot #1, sample preparation 2	2.4
Lot #1, sample preparation 3	2.1
Lot #1, sample preparation 4	2.2
Lot #2	2.1
Lot #3	2.2
Mean **Standard Deviation** **CV**	**2.20** **0.11** **5.0%**

***In Vitro* Neuropr tection Assay.** Neuronal precursor cells (either human or non-human) were subjected to conditions mimicking steps of *in vivo* neurodegeneration. Conditions were optimized to demonstrate a protective effect of the reference *Ginkgo biloba* material. Metabolic activity of treated cells in the presence and absence of the herbal reference material was compared to that of untreated cells.

Amyloid β-peptide (25-35) was obtained from Bachem Bioscience Inc. Peptide was dissolved in DMSO at 1 mM final concentration and aliquoted under N_2 atmosphere and kept at -30°C. A fresh aliquot was used in each experiment and diluted to the required concentration into culture medium just before use. Glutamate was purchased from Sigma (St. Louis, MO). A 10 mM stock was prepared in 18 MΩ pure water and sterile filtered. Aliqouts were kept at -30°C and diluted into culture medium before use.

The PC-12 precursor neuronal cell line (rat adrenal pheochromocytoma) was obtained from ATCC (Rockville, MD) and cultured in DMEM medium containing 10 % fetal bovine serum (FBS) and 5% heat inactivated horse serum in the presence of 1% penicillin/streptomycin solution. Heat inactivated horse serum was from Sigma (St. Louis,MO). Other tissue culture reagents were from Gibco. Cells were grown on collagen coated tissue culture surfaces. The fibrillar form of collagen (Cell Prime Collagen, Cohesion Technologies, Inc., Palo Alto, CA) was prepared according to the manufacturer's instruction and used to coat the tissue culture flasks and 96 wells plates in a form of a thin layer. Cells were dislodged from the plate by trituration. Cell clusters were broken up by vigorous pipetting and the cell suspension was filtered through a cell strainer to obtain even cell number and seeded at a cell density of 10^4/well 24 hours before experiments. Cells were pretreated with the *Ginkgo biloba* reference material that was dissolved in DMSO at 10 mg/ml concentration and diluted into culture medium at the required concentration just before use.

Table 13 shows cytotoxicity to PC12 cells of glutamate, amyloid-β peptide and a combination of both. The most dramatic decline in viability (% surviving cells) occurs from 48 to 72 hours. By 96 hours, cell numbers have started to increase as remaining viable cells replicate.

Table 13 - Neurotoxicity of Glutamate and Amyloid-β Peptide: Cell Viability (%)			
Time (hours)	**10 mM Glutamate**	**0.5 μM Amyloid-β Peptide**	**10 mM Glutamate + 0.5 μM Amyloid-β Peptide**
48	71	47	33
72	59	33	18
96	50	60	33

Tables 13 shows the dose-response relationship of a *Ginkgo biloba* reference material added to the culture and the resulting reduction in cellular toxicity of glutamate, amyloid-β peptide and a combination of both. Cell viability was determined 48 hours post-treatment (Table 14) and 72 hours post-treatment (Table 15). In all cases, addition of the reference material resulted in increased viability.

Table 14 - Protection from Neurotoxicity by *Ginkgo biloba* Reference Material: PC12 Cell Viability (%) 48 Hours Post-Treatment			
***Ginkgo biloba* Concentration (μg/ml)**	**10 mM Glutamate**	**0.5 μM Amyloid-β Peptide**	**10 mM Glutamate + 0.5 μM Amyloid-β Peptide**
0	75	43	35
25	84	51	43
50	92	55	52
100	102	66	61

Table 15 - Protection from Neurotoxicity by *Ginkgo biloba* Reference Material: PC12 Cell Viability (%) 72 Hours Post-Treatment			
***Ginkgo biloba* Concentration (μg/ml)**	**10 mM Glutamate**	**0.5 μM Amyloid-β Peptide**	**10 mM Glutamate + 0.5 μM Amyloid-β Peptide**
0	64	40	25
25	70	51	37
50	79	54	43
100	86	65	52

The ability of the Ginko biloba reference material to protect serum starved cells challenged with amyloid-β peptide was also investigated. Table 16 shows the further reduction in viability to cells cultured with two concentrations of amyloid-β peptide which results from serum starvation. The protective effect of the *Ginkgo biloba* reference material extends to serum starved cells. This assay is used to test *Ginkgo biloba* preparations resulting from simulated digestion and/or absorption for neuroprotective effectiveness.

Table 16 - Protection from Neurotoxicity and Serum Starvation by *Ginkgo biloba* Reference Material: PC12 Cell Viability (%)				
	***Ginkgo biloba* Extract Concentration (μg/ml)**	**Serum**	**0.5 μM Amyloid-β Peptide**	**1.0 μM Amyloid-β Peptide**
48 hours	0	-	41	41
	50	-	69	58
	0	+	49	47
	50	+	80	66
72 hours	0	-	35	36
	50	-	50	49
	0	+	47	53
	50	+	80	77

Example 4

***In Vitro* Assay for Biological Activity of St. John's Wort Preparations.** St. John's Wort (*Hypericum perforatum*) extracts and compositions were prepared using a simulated digestion model as described above. To reduce degradation of active constituents, incubations for gastric digestion and intestinal digestion were each conducted for one hour. The resulting preparations are evaluated *in vitro* for ability to inhibit ^{3}H-dopamine and ^{3}H-5-HT (serotonin) re-uptake in rat cortex synaptosomes. The assays exemplified are relevant to the mode of action of anti-depressants in general, and to the suspected mechanism of action of St. John's Wort formulations.

Synaptosome Preparation. Rat brain synaptosomes were prepared from striata for ^{3}H-dopamine uptake or from whole brain minus cerebellum for ^{3}H-5-HT uptake according to the method of Gray and Whittaker (1962). Briefly, male Sprague-Dawley rats were decapitated and the brains were rapidly removed. Either striata or whole brain minus cerebellum was weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose solution using a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 1,000 g at 0-4°C for 10 minutes. The supernatant was decanted and used for uptake experiments.

^{3}H-dopamine and ^{3}H-5-HT uptake. Uptake was measured as described by Bonnet *et al.* (1986). 50 μl aliquots of the crude synaptosomal preparations were incubated at 37°C in 1-2.5 mL of incubation medium of the following composition: 109 mM NaCl, 3.55 mM KCl, 2.4 mM $CaCl_2$, 0.61 mM $MgSO_4$, 1.1 mM KH_2PO_4, 25 mM $NaHCO_3$, 5.4 mM glucose, 0.025 mM nialamide, pH 7.4 (this medium is gassed with 95% O_2, 5% CO_2, 30 minutes prior to use) with either 20 nM ^{3}H-dopamine or 20 nM ^{3}H-5-HT. An incubation period of 5 minutes was employed. Uptake was stopped by dilution with 1.5 mL of ice-cold medium followed immediately by filtration under reduced vacuum through Whatman GF/B glass fiber filters. The filters were washed twice with 3 mL of ice-cold medium and dried overnight at room temperature. After successive additions to the filters of 0.5 mL of distilled water for 30 minutes, 3 mL ethylene glycol, and 5 mL Ready Safe® (Beckman) scintillant cocktail, ^{3}H-radioactivity was measured using an LS 6500 TA scintillation counter (Beckman). Specific uptake of ^{3}H-dopamine and ^{3}H-5-HT is defined as the difference between the total

uptake at 37°C and uptake at 4°C. GBR 12783 and imipramine were used as reference compounds for the ^{3}H-dopamine and ^{3}H-5-HT uptake assays, respectively. Stock solutions (10 mg/mL) prepared in DMSO were centrifuged at 17,000 g for 10 minutes and the supernatants were used in the assays. Samples were stored at 4°C for a maximum of about 5 days in amber colored bottles. Further dilutions were made in incubation medium.

Table 17 shows the inhibition of serotonin re-uptake for a reference St. John's Wort composition, as well as for a composition which has been subjected to gastric digestion alone or to gastric and intestinal digestion. The data represents the mean radioactivity counts of four replicate readings per sample and student's T-test statistical analysis (P) for significant activity versus the matched control. All St. John's Wort samples demonstrated significant serotonin re-uptake inhibition. However, compared to the reference St. John's Wort product dissolved in DMSO, inhibition was noticeably reduced where the St, John's Wort product had been subjected to simulated gastric digestion. Simulated intestinal digestion did not further reduce the inhibitory activity significantly.

Table 17 - Inhibition of Seratonin Reuptake by St. John's Wort

Sample	Average Counts/Minute	P Value Versus Appropriate Contol
Solvent Blank	31,529	
Reference St. John's Wort in DMSO	13,312	P<0.01
Simulated Gastric Fluid (SGF)	29,429	
Reference St. John's Wort (~88 μg/mi in SGF)	24,597	P<0.01
Simulated Gastric Fluid / Simulated Intestinal Fluid (SGF/SIF)	31,810	
Reference St. John's Wort (~88 μg/ml in SGF/SIF)	26,225	P<0.01

Table 18 shows the dose response relationship of the digested St. John's Wort reference and reduced serotonin re-uptake. The data represents the mean radioactivity counts of four replicate readings per sample and student's T-test statistical analysis (P) for significant activity versus the matched control. Similar tests of inhibition of dopamine re-uptake can be performed.

Table 18 - Dose Response of Inhibition of Seratonin Re-uptake by St. John's Wort

Sample	Average Counts / Minute	P Value Versus Appropriate Control
Solvent Blank (DMSO)	32,184	-
Reference St. John's Wort in DMSO	16,728	<0.01
Simulated Gastric Fluid / Simulated Intestinal Fluid (SGF/SIF)	37,785	-
Reference St. John's Wort (SGF/SIF) (400 μg/ml)	23,104	<0.01
Reference St. John's Wort (SGF/SIF) (200 μg/ml)	28,860	<0.01
Reference St. John's Wort (SGF/SIF) (100 μg/ml)	34,924	0.08

Example 5

***In Vitro* Assays of Biological Activity of Saw Palmetto Preparations.** Assays for inhibition of 5α-reductase or cyclooxygenase are useful for measuring activity of Saw Palmetto compositions prepared using the simulated digestion and absorption models described above as well as for other Saw Palmetto extracts and commercial products.

Preparation of Rat Liver Microsomes and 5α-reductase enzyme. Male Sprague-Dawley rats (Hilltop Lab Animal, Scottdale, PA) were anesthetized using CO_2 gas. Connective tissue surrounding the liver was removed and the liver was perfused two times with HEDG perfusion buffer (Hepes pH 7.2, 1.5 mM EDTA, 1.0 mM DTT and 10% Glycerol). The liver was then removed and homogenized in HEDG buffer (5 ml buffer per gram of wet liver

weight). The homogenate was centrifuged at 9000 x g for 20 minutes at 4°C. The resulting supernatant was then centrifuged at 100,000 x g for one hour at 4°C. The resulting microsomal pellet was resuspended in HEDG and homogenized (3 strokes at 100 rpm), aliqoted, flash frozen, and stored at -80°C.

Assay for inhibition of 5α-reductase activity by detection of androgen metabolism. For each saw palmetto sample to be tested, the material was diluted to the desired concentration (50 μg/ml saw palmetto extract) in 20 mM potassium phosphate buffer, pH 7.0. 20 μl of microsomes (enzyme) followed by testosterone (25 μg/ml final concentration) and progesterone (20 μg/ml, final concentration). 5α-reductase substrates were then added to reaction mixture. The enzymatic reaction was initiated with the addition of NADPH (490 μg/ml final concentration) and was incubated at 37°C for one hour. The samples were then prepared for HPLC analysis by ether extraction of substrates. Testosterone and progesterone peak areas were integrated, compared to testosterone and progesterone standards, and inhibition was calculated based on the amount of unmetabolized substrate remaining.

Initially, it was found that 5α-reductase activity is inhibited to a large extent by components of the digestion buffer and placebo capsules. However, as shown in Table 19, boiling for 2 minutes or heating of the digestion products to 50°C for five minutes was effective for reducing non-specific inhibition of 5α-reductase by those components, while the inhibitory activity contained in Saw Palmetto preparations remained intact. The data in Table 19 represent the mean percent inhibition of testosterone metabolism when incubated with 5α-reductase in the presence of 50 μg/ml Saw Palmetto preparations.

These data show that Saw Palmetto materials are active after simulated digestion even after inactivation of intestinal enzymes by boiling or heating. Samples which were filtered through 0.2μ syringe filters lost activity (data not shown).

Table 19 - Inhibition of 5α-reductase after Heat Treatment of Digested Materials		
Sample	**Treatment**	**Percent Inhibition**
Saw Palmetto Reference	-	59%
Saw Palmetto Reference	Boiled	42%
Saw Palmetto Reference	50°C	55%
Placebo	Boiled	0%
Placebo	50°C	10%

Assay for inhibiton of 5α-reductase by detection of formation of 5HT and transformation of androgen receptor. A single multi-site assay is capable of modeling, *in vitro*, synergistic activity between inhibition of 5HT formation and inhibition of 5HT transformation of androgen receptor. The following mixture is used to assay for biological activity: recombinant 5-alpha reductase or microsomes containing 5-alpha reductase, cofactor NADPH, substrate testosterone in limiting concentration, cytosol or nuclear extract containing competent unliganded androgen receptor bound to HSP90, and biotinylated androgen response element DNA in limiting concentration. Other components may be added to the above listed components, where appropriate.

Aliquots of the mixture are challenged with a Saw Palmetto composition, a positive control comprising a similarly prepared sample lacking the Saw Palmetto composition, and a negative control lacking both testosterone and the Saw Palmetto composition. **[The Saw Palmetto composition is ...]** The assay detects the inhibition of the formation of 5HT from testosterone which is catalyzed by 5-alpha-reductase. 5HT binds to and transforms androgen receptor, such that androgen receptor can bind to androgen response element DNA. Thus, by inhibiting the formation of 5HT, Saw Palmetto inhibits the transformation of androgen receptor into the form which is capable of binding to androgen response element DNA. To detect whether androgen receptor has been transformed, the mixture is contacted with neutravidin, avidin, or streptavidin bound to an ELISA plate, whereby the biotinylated

androgen response element DNA of the mixture is immobilized. Androgen receptor in the mixture, if transformed by 5HT, forms a complex with the androgen response element DNA and is thus immobilized. Unbound materials are washed away. The ELISA plate is contacted with anti-androgen receptor antibody, such that the antibody binds to the androgen response element-androgen receptor complex on the plate. The antibody and complex may be detected using any number of techniques known in the art. For example, the antibody may be labeled with an enzyme or a fluorescent tag or other detectable moiety. In the preferred method, a second antibody, i.e. anti-antibody coupled to alkaline phosphatase enzyme, is bound to the antibody and complex bound to the plate. Alkaline phosphatase is detected enzymatically using para nitrophenyl phosphate (PNPP) and measured colorimetrically in an ELISA plate reader. The activity of the Saw Palmetto containing reaction less the negative control divided by positive control less the negative control gives percent inhibition of the Saw Palmetto extract, and several doses within the range of the assay are used to estimate the IC_{50}.

Assay for inhibition of production of PGE_2. Compositions of Saw Palmetto are tested for their ability to inhibit prostaglandin E_2 (PGE_2) production by macrophage cells stimulated with interferon-γ (IFN-γ).

RAW264.7 macrophage cells (ATCC, Rockville, MD) were propagated in DMEM (Gibco, Long Island, NY) supplemented with 2 mM L-glutamine (Gibco), 10% fetal bovine serum (Gemini, Carlsbad, CA) and 1% penicillin/streptomycin solution. Cells were passaged at a cell density of 10^6 cells/ml and used at this same cell number for all experiments. RAW264.7 cells were inoculated into 24-well tissue culture plates (1 ml/well). Test cells were incubated with and without Saw Palmetto preparations prepared by simulated digestion as in Example 1, and simultaneously stimulated for induction of PGE_2 production with 10 units/ml IFN-γ (Gibco). Indomethacin (Sigma, St. Louis, MO), a known synthetic cyclooxygenase inhibitor, was used as a positive control for this methodology. The amount of PGE_2 produced was quantified by assaying PGE_2 levels in the tissue culture media using a quantitative PGE_2 ELISA Kit (Oxford Biomedical Research, Oxford, MI) in conjunction with a standard curve containing known PGE_2 concentrations diluted into tissue culture media.

Table 20 shows the dose response relationship for three concentrations of digested Saw Palmetto reference material or indomethacin and PGE_2 production by RAW264.7 macrophages stimulated with IFN-γ. We next evaluated the lot-to-lot reproducibility of a Saw Palmetto product standard. Three lots of the Saw Palmetto standard were subjected to simulated digestion and assayed on the same day at the same concentration of 20 μg/ml (Table 21).

Table 20 - Inhibition of PGE_2 Production by Indomethacin and Digested Saw Palmetto Reference

Indomethacin Concentration (μg/ml)	Indomethacin Response (% Inhibition)	Saw Palmetto Concentration (μg/ml)	Saw Palmetto Response (% Inhibition)
0.01	74.8	40	73.8
0.001	53.9	20	47.7
0.0001	41.8	10	18.8

Table 21 - Inhibition of PGE_2 Production by Preparations of Three Saw Palmetto Reference Lots

Sample	% PGE_2 Inhibition	Std. Deviation	%CV
Saw Palmetto Lot#1 (20 μg/ml)	48.1	-	-
Saw Palmetto Lot#2 (20 μg/ml)	48.9	-	-
Saw Palmetto Lot#3 (20 μg/ml)	57.5	-	-
Mean Responses	**51.5**	**5.2**	**10.2**

Example 6

***In vitro* assay for biological activity of *Panax ginseng* preparations.** Assays for biological activity are based on induction of corticsteroids and glucocorticoid modulation of nitric oxide synthase isozymes.

Assay for corticosterone induction. Swiss mice (20-25 grams), supplied through Hilltop Lab Animal (Scottdale, PA), were housed in polycarbonate cages with wood-shaving bedding with a 12 hour day-light cycle at 72°C. Animals were supplied rodent feed and water *ad libitum*. Corticosterone I^{125} radioimmunoassay (RIA) kits were supplied through ICN Biomedicals (Costa Mesa , CA) and used in accordance with manufacturer's instructions. The ginseng preparation used for the development and validation of this assay was a product containing a clinically tested and proven ginseng extract (G115®).

Test samples were prepared in phosphate buffered saline (PBS) to a concentration of 60 mg/ml or subjected to the simulated digestion protocol as described in Example 1, with the exception that the amount of ginseng test material used yielded a final concentration of ginseng extract of 50 mg/ml. The test material(s) or PBS vehicle control were administered by intraperitoneal injection at a dose of 50 mg ginseng extract/100 gram animal body weight using 5 animals/treatment group. One hour post-dosing blood plasma was produced and levels of corticosterone were determined using the quantitative RIA. Statistical analysis was via students T-test. Table 22 shows typical results obtained with these procedures. The statistical significance (P) is relative to the saline control. The data demonstrate that the corticosterone activity survives simulated digestion, and is functional *in vivo*.

Table 22 - Gensing Induced Corticosteroid Production

Test Sample	Corticosteroid produced ng/ml	P value
Control	62.7	-
Ginseng in PBS	427.3	0.002
Digested Ginseng	450.8	<0.001

Assay f r inhibition of nitric oxide production. RAW264.7 macrophage cells (ATCC, Rockville, MD) were propagated in DMEM (Gibco, Long Island, NY) supplemented with 2 mM L-glutamine (Gibco), 10% fetal bovine serum (Gemini, Carlsbad, CA) and 1% penicillin/streptomycin solution. Cells were passaged at a cell density of 10^6 cells/ml and used at this same cell number for all experiments. RAW264.7 cells were inoculated into 96-well tissue culture plates (100 μl/well). Test cells were incubated with and without ginseng preparations prepared by simulated digestion as in Example 1, and simultaneously stimulated for induction of nitric oxide production with 10 units/ml IFN-γ **[Source?]**. Progesterone (Sigma, St. Louis, MO) was used as a positive control. (Miller *et al.*, 1996). The amount of nitric oxide produced was quantified by assaying for nitric oxide by-product, nitrites in the tissue culture media using the Griess reagent (Sigma, St. Louis, MO) (Miller *et al.*, 1996) in conjunction with a standard curve containing known micromolar amounts of nitrites in tissue culture media.

Table 23 shows the dose-response relationship for three different doses between digested Ginseng material or progesterone control and inhibition of production of nitric oxide. To ensure that this activity was not due to direct free radical scavenging, digested ginseng preparations were also tested by the method described in Example 3. Doses up to 400 μg/mL displayed no direct free radical scavenging activity indicating that reduction in levels of nitric oxide was most likely due to inhibition of nitric oxide production.

Table 23 - Inhibition of Nitric Oxide Production by Progesterone and Ginseng Reference

Progesterone Concentration (μg/ml)	% Inhibition	Ginseng Extract Concentration (μg/ml)	% Inhibition
100	96.7	400	87.6
50	79.0	200	31.7
25	33.3	100	21.6

Table 24 shows the lot-to-lot reproducibility of inhibition of nitric oxide production for a set concentration of digested ginseng reference material of 200 μg/ml. The 5 lots tested showed some variability in activity, but overall this variability was low < 10% (based on relative standard deviation, %CV).

Table 24 - Inhibition of Oxide Production by Ginseng Reference

Sample	Response (μM)	% Inhibition	Standard Deviation	%CV
Placebo	27.0	-	0.71	2.6
Ginseng Lot #1	-	51.8	0.75	1.4
Ginseng Lot #2	-	51.9	0.42	0.8
Ginseng Lot #3	-	46.2	0.90	1.9
Ginseng Lot #4	-	42.8	0.79	1.8
Ginseng Lot #5	-	43.5	1.46	3.4
Overall	-	47.2	4.15	8.8

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What Is Claimed Is:

1. A method for evaluating the biofunctionality of a natural composition or extract comprising:

subjecting said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said natural composition;

determining the presence or absence of one or more components or biological activities in said preparation; and

correlating the presence or absence of said one or more components or biological activities in said preparation to the biofunctionality of said natural composition or extract.

2. A method for evaluating the biofunctionality of a natural composition or extract comprising:

subjecting said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said natural composition;

determining the presence or absence of one or more components in said preparation; and

correlating the presence or absence of said one or more components to the biofunctionality of said natural composition or extract

3. A method for evaluating the biofunctionality of a natural composition or extract comprising:

subjecting said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said natural composition;

determining the presence or absence of one or more biological activities in said preparation; and

correlating the presence or absence of said one or more biological activities in said preparation to the biofunctionality of said natural composition or extract

4. The method of Claim 1 which comprises subjecting said natural composition to simulated digestion and to simulated absorption.

5. The method of Claim 4 wherein said simulated digestion and said simulated absorption are carried out sequentially.

6. The method of Claim 4 wherein said simulated digestion and said simulated absorption are carried out concurrently.

7. The method of Claim 1 wherein said simulated digestion comprises a gastric digestion or an intestinal digestion.

8. The method of Claim 1 wherein said simulated digestion comprises a gastric digestion and an intestinal digestion.

9. The method of Claim 7 wherein said gastric digestion comprises subjecting the natural composition to simulated gastric fluid for an effective amount of time to digest the natural composition or extract.

10. The method of Claim 9 wherein said simulated gastric fluid comprises at least one gastric enzyme.

11. The method of Claim 9 wherein said simulated gastric fluid contains pepsin and has a pH between about 1.0 and about 2.0.

12. The method of Claim 9 wherein said simulated gastric fluid contains pepsin and has a pH of approximately 1.2.

13. The method of Claim 7 wherein said intestinal digestion comprises subjecting the natural composition to simulated intestinal fluid for an effective amount of time to digest the natural composition or extract.

14. The method of Claim 13 wherein said simulated intestinal fluid comprises at least one pancreatic enzyme selected from the group consisting of pancreatin, trypsin, chymotrypsin, carboxypeptidase and elastase.

15. The method of Claim 1 wherein said simulated absorption comprises transport of one or more components of said composition across a biological membrane.

16. The method of Claim 1 wherein said simulated absorption comprises transport of one or more components of said composition from the apical side to the basolateral side of a cell monolayer.

17. The method of Claim 16 wherein said cell monolayer comprises Caco-2 cells.

18. The method of Claim 1 wherein said natural composition is derived from a botanical source.

19. The method of Claim 1 wherein said natural composition is derived from an herbal source.

20. The method of Claim 1 wherein said natural composition is derived from an animal source.

21. The method of Claim 1 wherein said natural composition comprises *Echinacea* or an extract thereof.

22. The method of Claim 21 wherein the presence or absence of biological activity is determined by measuring immunostimulatory activity of said preparation.

23. The method of Claim 22 which further comprises:
providing a mixture of said preparation and T lymphocytes; and

measuring the amount of Tumor Necrosis Factor present in said mixture after a predetermined incubation period.

24. The method of Claim 22 which further comprises:
providing a mixture of said preparation and a macrophage cell line; and
measuring the amount of Tumor Necrosis Factor present in said mixture after a predetermined incubation period.

25. The method of Claim 24 wherein the macrophage cell-line is RAW264.7.

26. The method of Claim 21 wherein the presence of absence of biological activity is determined by measuring proliferation of peripheral blood mononuclear cells (PBMCs).

27. The method of Claim 26 wherein measuring proliferation comprises measuring the conversion of the metabolic dye MTT.

28. The method of Claim 1 wherein said natural composition comprises St. John's Wort or an extract thereof.

29. The method of Claim 28 wherein the presence or absence of biological activity is measured by:
providing a mixture containing said preparation, a synaptosomal preparation and a neurotransmitter selected from the group consisting of dopamine and serotonin; and
detecting uptake of said neurotransmitter by the synaptosomal preparation in said mixture.

30. The method of Claim 1 wherein said natural composition comprises saw palmetto or an extract thereof.

31. The method of Claim 30 wherein the biological activity is measured by:

providing a mixture comprising said preparation, microsomes, cofactor NADPH, and one or more; and

detecting metabolism of one or more of said substrates.

32. The method of 31 wherein said one or more 5-alpha-reductase substrates, are testosterone and progesterone.

33. The method of Claim 30 wherein the biological activity is measured by:

providing a mixture comprising said preparation, 5-alpha-reductase, cofactor NADPH, testosterone, unliganded androgen receptor complexed with HSP90 and androgen response element DNA; and

detecting formation of an androgen receptor-androgen response element complex in said mixture.

34. The method of Claim 33 wherein androgen receptor-androgen response element complex is immobilized on a solid support.

35. The method of Claim 33 wherein said androgen receptor-androgen response element complex is detected colorimetrically or by fluorescence.

36. The method of Claim 30 wherein the biological activity is determined by:

providing a mixture comprising said preparation, unliganded aryl hydrocarbon receptor and aryl hydrocarbon response element DNA; and

detecting formation of an aryl hydrocarbon receptor-aryl hydrocarbon response element DNA complex in said mixture.

37. The method of Claim 36 wherein said androgen receptor-androgen response element complex is immobilized on a solid support.

38. The method of Claim 36 wherein said androgen receptor-androgen response element complex is detected colorimetrically or by fluorescence.

39. The method of Claim 30 wherein the biological activity is determined by:

providing a mixture comprising said preparation, macrophages and interferon-γ (IFN-γ); and

detecting activation of said macrophages.

40. The method of Claim 39 wherein detecting activation of said macrophages comprises detecting production of prostaglandin E_2 (PGE_2) or nitric oxide.

41. The method of Claim 1 wherein said natural composition comprises *Ginkgo biloba* or an extract thereof.

42. The method of Claim 41 wherein the biological activity is determined by measuring free radical scavenging activity.

43. The method of Claim 41 wherein the biological activity is determined by measuring the amount of inhibition of platelet activating factor.

44. The method of Claim 41 wherein the biological activity is determined by measuring inhibition of neurotoxicity.

45. The method of Claim 44 wherein measuring inhibition of neurotoxicity comprises:

subjecting a neuronal cell in the presence of said preparation to a substance or condition effective to reduce neuronal cell viability; and

measuring the viability of said neuronal cell.

46. The method of Claim 1 wherein said natural composition comprises *Panax ginseng* or an extract thereof.

47. The method of Claim 46 wherein the biological activity is determined by:

administering said preparation to a test subject; and

measuring the amount of corticosterone production in said test subject.

48. The method of Claim 46 wherein the biological activity is determined by:

providing a mixture comprising said preparation, macrophages and interferon-γ (IFN-γ); and

measuring the amount of inhibition of nitric oxide production in said macrophages.

49. A dietary supplement comprising a natural composition, having substantial batch-to-batch compositional consistency, said compositional consistency evaluated by a method comprising:

subjecting a sample of said dietary supplement or said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said sample;

determining the presence of one or more components in said preparation; and

correlating the presence of one or more components with compositional consistency.

50. A dietary supplement comprising a natural composition, having substantial batch-to-batch compositional consistency, said compositional consistency evaluated by a method comprising:

subjecting a sample of said dietary supplement or said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said sample;

determining the presence of one or more components in said preparation; and

correlating the presence of one or more components with compositional consistency.

51. A dietary supplement comprising a natural composition, having substantial batch-to-batch compositional consistency, said compositional consistency evaluated by a method comprising:

subjecting a sample of said dietary supplement or said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said sample;

determining the presence of one or more biological activities in said preparation; and

correlating the presence of one or more biological activities with compositional consistency.

52. The dietary supplement of Claim 49 wherein said dietary supplement or said natural composition is subjected to simulated digestion and simulated absorption.

53. The dietary supplement of Claim 52 wherein said simulated digestion and said simulated absorption are carried out sequentially.

54. The dietary supplement of Claim 52 wherein said simulated digestion and said simulated absorption are carried out concurrently.

55. The dietary supplement of Claim 49 wherein said simulated digestion comprises a gastric digestion or an intestinal digestion.

56. The dietary supplement of Claim 49 wherein said simulated digestion comprises a gastric digestion and an intestinal digestion.

57. The dietary supplement of Claim 55 wherein said gastric digestion comprises subjecting said dietary supplement or said natural composition to simulated gastric fluid for an effective amount of time to digest the natural composition or extract.

58. The dietary supplement of Claim 57 wherein said simulated gastric fluid comprises a gastric enzyme.

59. The dietary supplement of Claim 57 wherein said simulated gastric fluid comprises pepsin and has a pH between about 1.0 and about 2.0.

60. The dietary supplement of Claim 57 wherein said simulated gastric fluid contains pepsin and has a pH of approximately 1.2.

61. The dietary supplement of Claim 55 wherein said intestinal digestion comprises subjecting said dietary supplement or said natural composition to simulated intestinal fluid for an effective amount of time to digest the natural composition.

62. The dietary supplement of Claim 61 wherein said simulated intestinal fluid comprises at least one pancreatic enzyme selected from the group consisting of pancreatin, trypsin, chymotrypsin, carboxypeptidase and elastase.

63. The dietary supplement of Claim 49 wherein said simulated absorption comprises transport of one or more components of said composition across a biological membrane.

64. The dietary supplement of Claim 49 wherein said simulated absorption comprises transport of one or more components of said composition from the apical side to the basolateral side of a cell monolayer.

65. The dietary supplement of Claim 64 wherein said cell monolayer comprises Caco-2 cells.

66. The dietary supplement of Claim 49 wherein said natural composition is derived from a botanical source.

67. The dietary supplement of Claim 49 wherein said natural composition is derived from an herbal source.

68. The dietary supplement of Claim 49 wherein said natural composition is derived from an animal source.

69. The dietary supplement of Claim 49 wherein said natural composition comprises *Echinacea* or an extract thereof.

70. The dietary supplement of Claim 69 wherein the preparation is tested for immunostimulatory activity.

71. The dietary supplement of Claim 70, wherein compositional consistency is determined by:

providing a mixture of said preparation and T lymphocytes;

measuring the amount of Tumor Necrosis Factor present in said mixture after a predetermined incubation period; and

correlating the amount of Tumor Necrosis Factor with compositional consistency.

72. The dietary supplement of Claim 70 wherein compositional consistency is determined by:

providing a mixture containing said preparation and a macrophage cell line;

measuring the amount of Tumor Necrosis Factor present in said mixture after a predetermined incubation period; and

correlating the amount of Tumor Necrosis Factor with compositional consistency.

73. The dietary supplement of Claim 72 wherein the macrophage cell-line is RAW264.7.

74. The dietary supplement of Claim 69 wherein compositional consistency is determined by:

providing a mixture containing said preparation and peripheral blood mononuclear cells (PBMCs);

measuring proliferation of said PBMCs in said mixture; and

correlating proliferation of said PBMCs with compositional consistency.

75. The dietary supplement of Claim 74 wherein measuring proliferation comprises measuring the conversion ot the metabolic dye MTT.

76. The dietary supplement of Claim 49 wherein said natural composition comprises St. John's Wort or an extract thereof.

77. The dietary supplement of Claim 76 wherein compositional consistency is determined by:

providing a mixture containing said preparation, a synaptosomal preparation and a neurotransmitter selected from the group consisting of dopamine and serotonin;

detecting uptake of said neurotransmitter by the synaptosomal preparation; and

correlating the amount of uptake with compositional consistency.

78. The dietary supplement of Claim 49 wherein said natural composition comprises saw palmetto or an extract thereof.

79. The dietary supplement of Claim 78 wherein compositional consistency is determined by:

providing a mixture comprising said preparation, microsomes, cofactor NADPH, and one or more 5-alpha-reductase substrates;

detecting metabolism of one or more of said substrates; and

correlating metabolism of said substrates with compositional consistency.

80. The dietary supplement of Claim 79 wherein said one or more 5-alpha-reductase substrates are testosterone and progesterone.

81. The dietary supplement of Claim 78 wherein compositional consistency is determined by:

providing a mixture comprising said preparation, 5-alpha-reductase, cofactor NADPH, testosterone, unliganded androgen receptor complexed with HSP90 and androgen response element DNA;

detecting the formation of an androgen receptor-androgen response element complex; and

correlating formation of said complex with compositional consistency.

82. The dietary supplement of Claim 81 wherein said androgen receptor-androgen response element complex is immobilized on a solid support.

83. The dietary supplement of Claim 81 wherein said androgen receptor-androgen response element complex is detected colorimetrically or by fluorescence.

84. The dietary supplement of Claim 78 wherein compositional consistency is determined by:

providing a mixture comprising said preparation, unliganded aryl hydrocarbon receptor and aryl hydrocarbon response element DNA;

detecting the formation of an aryl hydrocarbon receptor-aryl hydrocarbon response element DNA complex; and

correlating formation of said complex with compositional consistency.

85. The dietary supplement of Claim 84 wherein said androgen receptor-androgen response element complex is immobilized on a solid support.

86. The dietary supplement of Claim 84 wherein said androgen receptor-androgen response element complex is detected colorimetrically or by fluorescence.

87. The dietary supplement of Claim 78 wherein compositional consistency is determined by:

providing a mixture comprising said preparation, macrophages and interferon-γ (IFN-γ);

detecting activation of said macrophages; and

correlating activation of said macrophages with compositional consistency.

88. The method of claim 87 wherein detecting activation of said macrophages comprises detecting production of prostaglandin E_2 or nitric oxide.

89. The dietary supplement of Claim 49 wherein said natural composition comprises *Ginkgo biloba* or an extract thereof.

90. The dietary supplement of Claim 89 wherein compositional consistency is determined by observing free radical scavenging activity and correlating said activity with compositional consistency.

91. The dietary supplement of Claim 89 wherein compositional consistency is determined by observing inhibition of platelet activating factor and correlating said inhibition with compositional consistency.

92. The dietary supplement of Claim 89 wherein compositional consistency is determined by observing inhibition neurotoxicity and correlating inhibition with compositional consistency.

93. The dietary supplement of Claim 92 wherein compositional consistency is determined by:

subjecting a neuronal cell in the presence of said preparation to a substance or condition effective to reduce neuronal cell viability;

measuring viability of said neuronal cell; and

correlating viability of neuronal cell with compositional consistency.

94. The dietary supplement of Claim 49 wherein said natural composition comprises *Panax ginseng* or an extract thereof.

95. The dietary supplement of Claim 94 wherein compositional consistency is determined by:

administering said preparation to a test subject;

measuring corticosterone production in said test subject; and

correlating corticosterone production with compositional consistency.

96. The dietary supplement of Claim 94 wherein compositional consistency is determined by:

providing a mixture comprising said preparation, macrophages and interferon-γ (IFN-γ); and

measuring inhibition of nitric oxide production in said macrophages; and

correlating inhibition of nitric oxide production with compositional consistency.

97. A process for making a dietary supplement comprising a natural composition, said dietary supplement having substantial batch-to-batch consistency, said compositional consistency evaluated by a method comprising:

subjecting a sample of said natural composition to simulated digestion, simulated absorption or both to obtain a preparation of said sample;

determining the presence or absence of one or more components in said preparation; and

selecting preparations comprising said one or more components.

98. A method for evaluating the biological activity of a *Ginkgo biloba* composition which comprises:

subjecting a neuronal cell in the presence of said composition to one or more substances or conditions effective to reduce viability of said neuronal cell; and

measuring the viability of said neuronal cell.

99. The method of claim 98 wherein said one or more substances are amyloid-β peptide or glutamate.

100. A method for evaluating the biological activity of a saw palmetto composition which comprises detecting inhibition of the transformation of androgen response receptor to DNA binding form which comprises:

providing a mixture containing said composition, 5HT, unliganded androgen receptor complexed to HSP90 and androgen response element DNA; and

detecting the formation of an androgen receptor-androgen response element DNA complex.

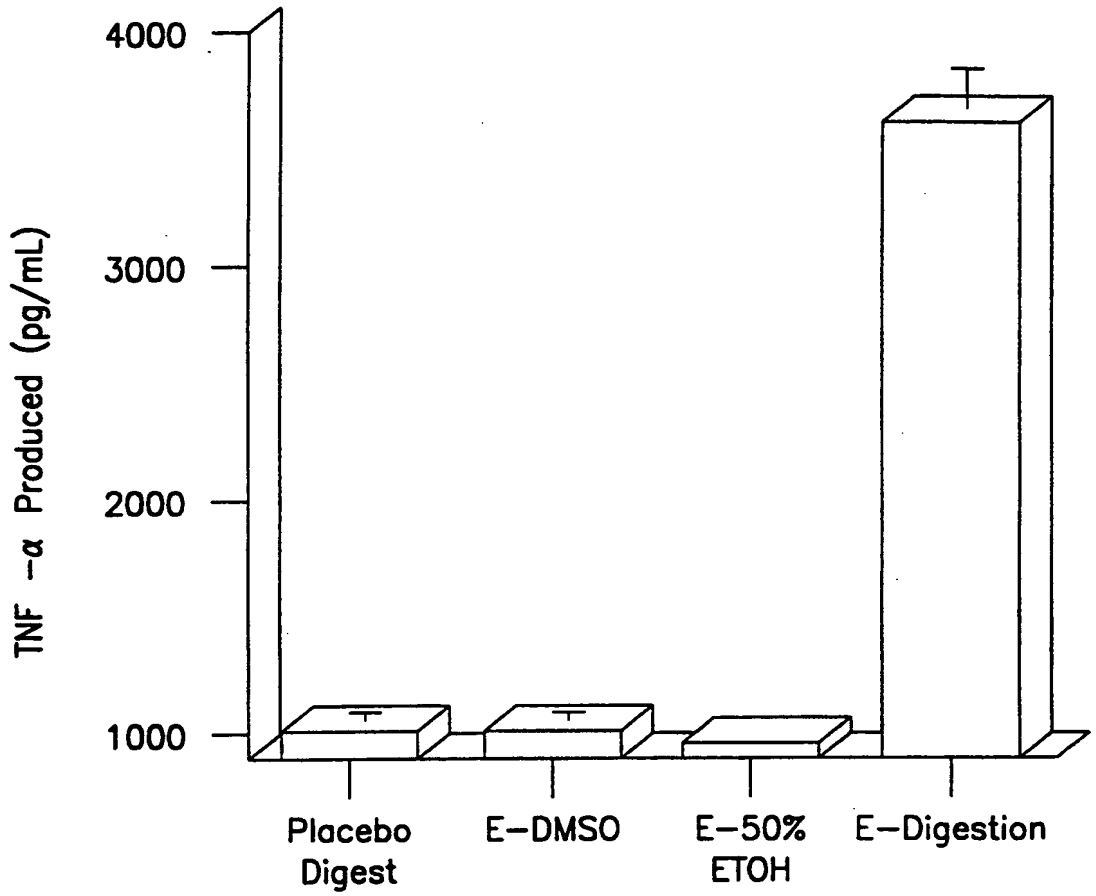
4000
3000
2000
1000
TNF -α Produced (pg/mL)
Placebo Digest
E-DMSO
E-50% ETOH
E-Digestion
Sample Preparation

Fig. 1

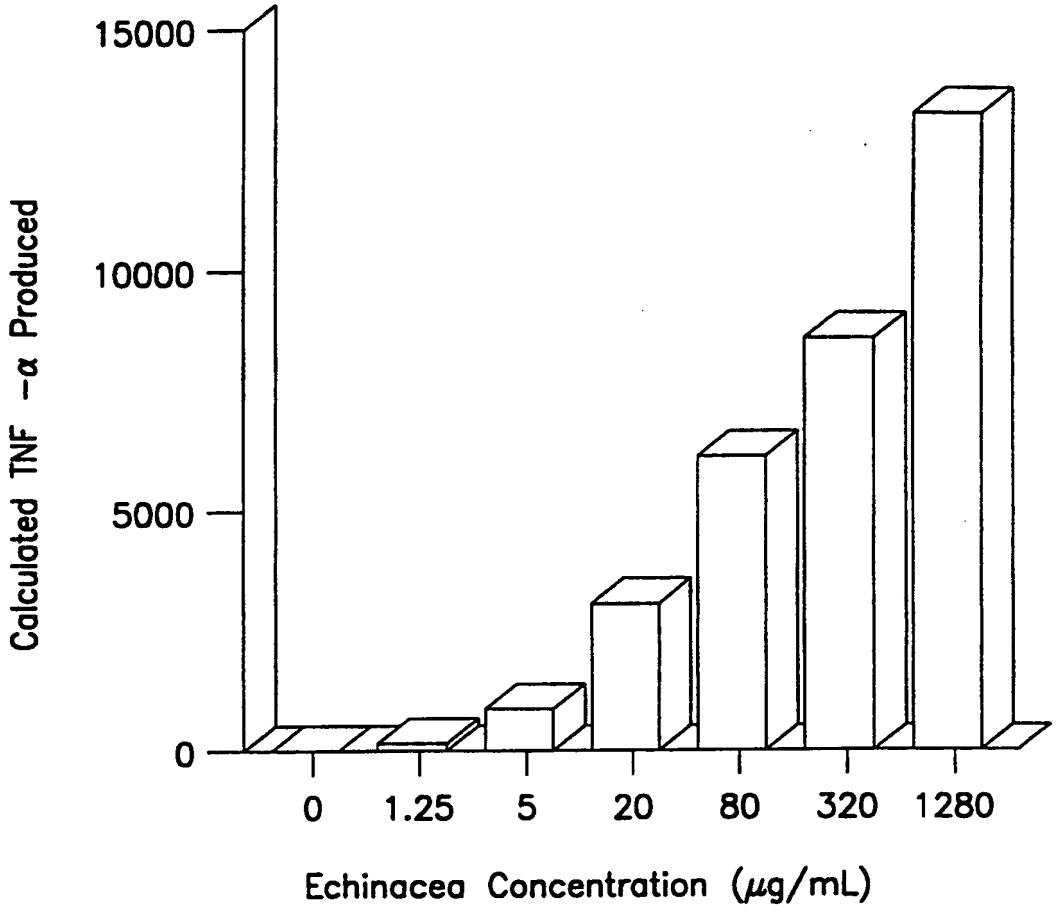
15000
10000
5000
0
Calculated TNF -α Produced
0
1.25
5
20
80
320
1280
Echinacea Concentration (μg/mL)

Fig. 2

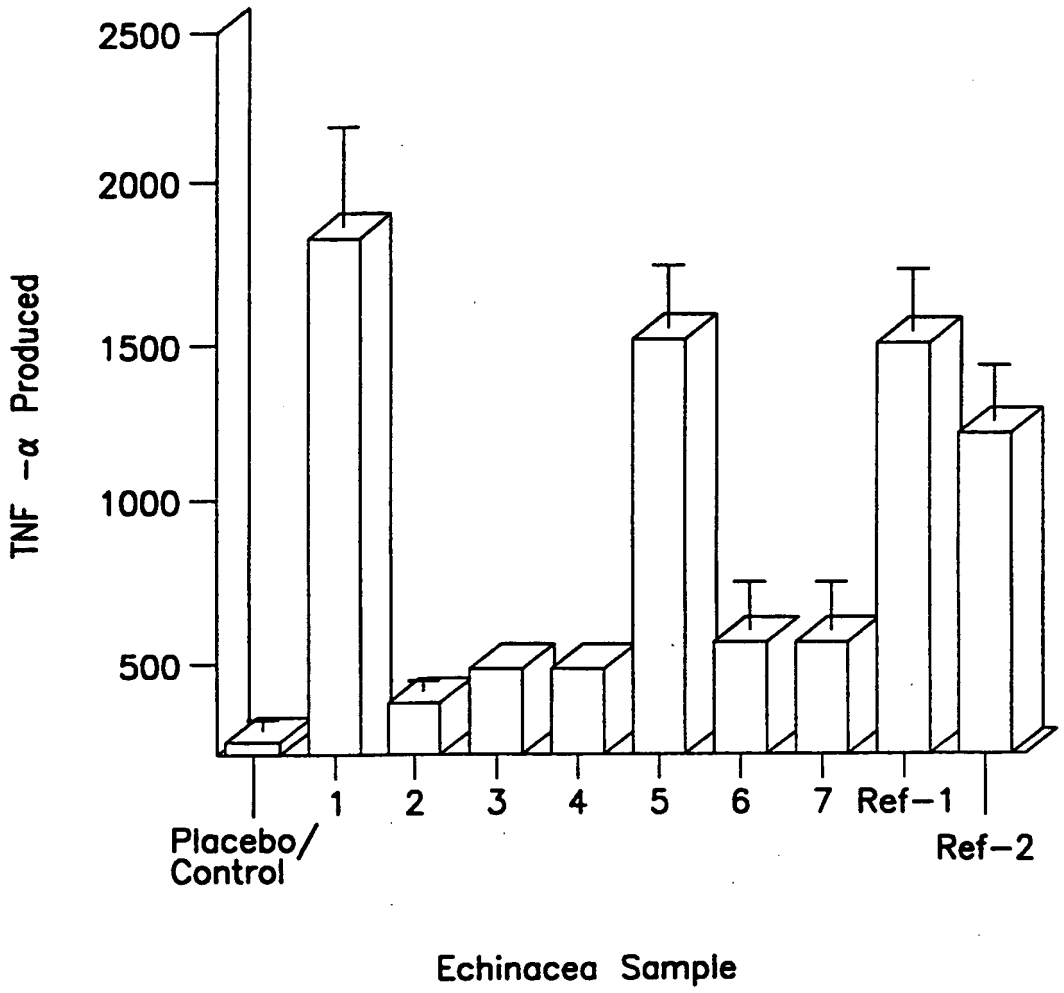
2500
2000
1500
1000
500
TNF -α Produced
Placebo/
Control
1
2
3
4
5
6
7
Ref-1
Ref-2
Echinacea Sample

Fig. 3

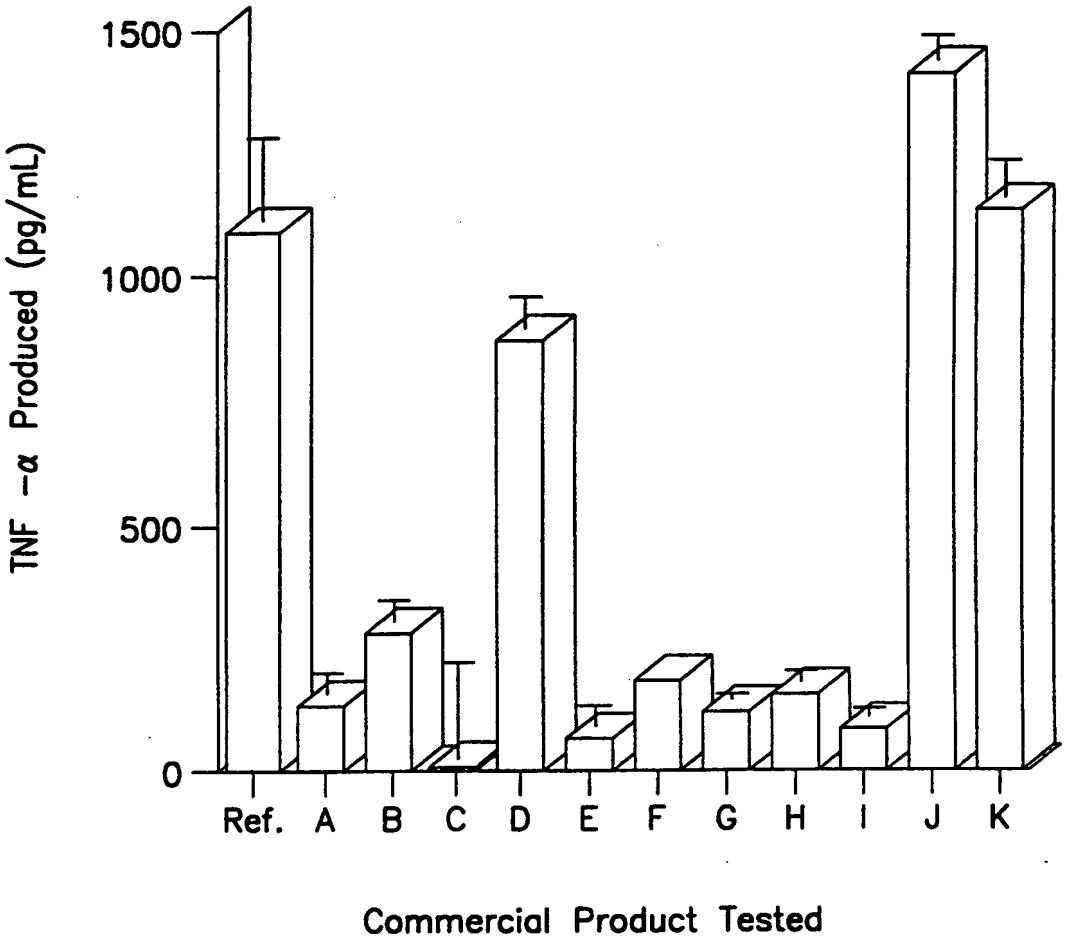
1500
1000
500
0
TNF -α Produced (pg/mL)
Ref.
A
B
C
D
E
F
G
H
I
J
K
Commercial Product Tested

Fig. 4

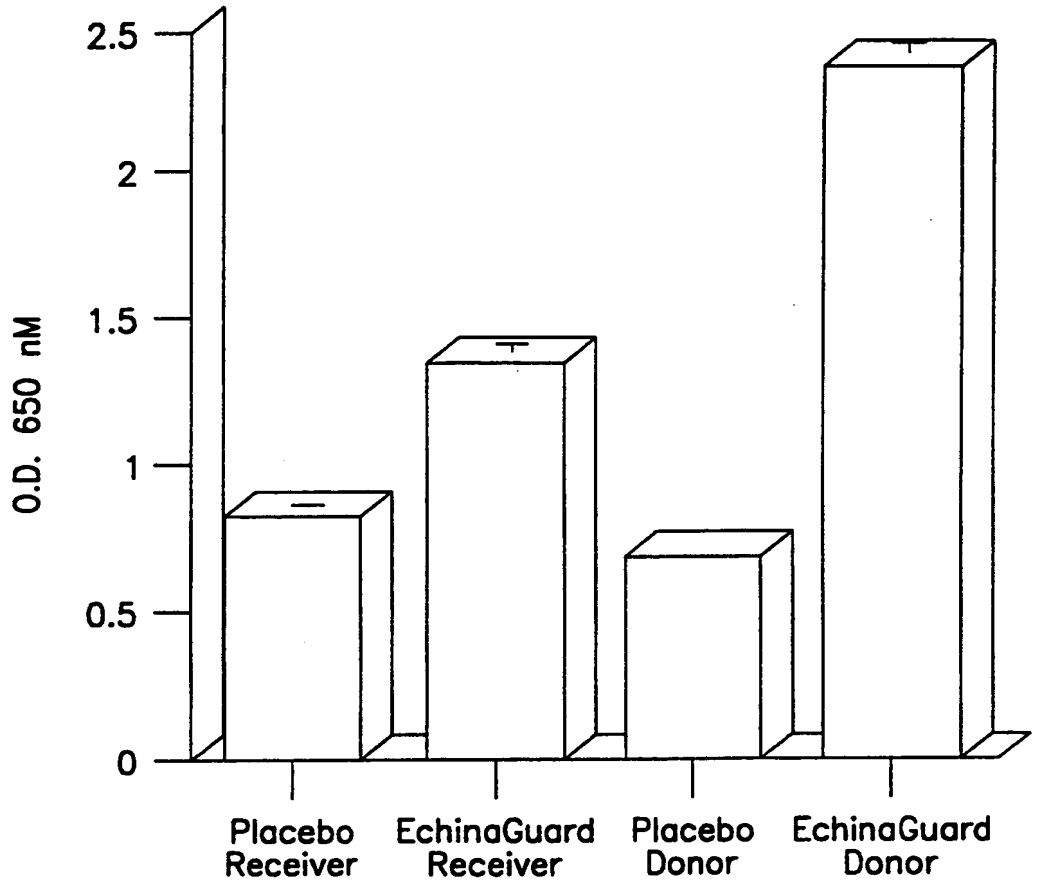
2.5
2
1.5
1
0.5
0
O.D. 650 nM
Placebo Receiver
EchinaGuard Receiver
Placebo Donor
EchinaGuard Donor
Caco-2 Sample

Fig. 5

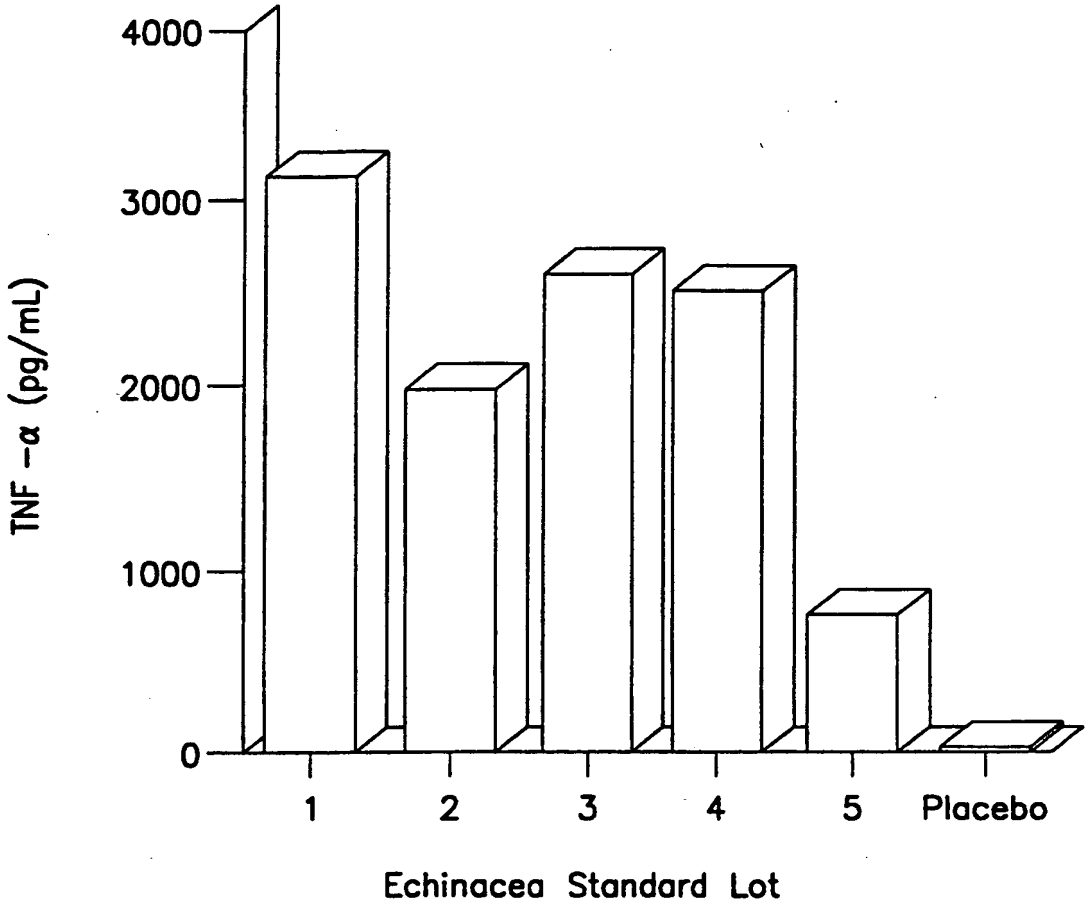
4000
3000
2000
1000
0
TNF -α (pg/mL)
1
2
3
4
5
Placebo
Echinacea Standard Lot

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15957

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 35/78
US CL :424 /195.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424 /195.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROSSI G. Biolgical Testing. Chapter 31 of Remington's Pharmaceutical Sciences, Philadelphia College of Pharmacy and Science, 16th Edition, 1980, pages 520-531.	1-100
Y	Hobbs C. Handbood for Herbal Healing. Botanica Press, Santa Cruz, CA, October 1990, pages 30-33.	1-100
Y	VAN BREEMEN R. Degradation of Peptide Drugs by Immobilized Digestive Proteases. Drug Metabolism and Disposition Vol, 19 No. 3, 1991 pages 683-690,	
Y	WALGREN R. Transport of Quercetin and its Glucosides Across Human Intestinal Epithelial Caco-2 Cells. Biochemical Pharmacology. 10 May 1998,Vol,55 No. 10 pages 1721-1727.	1-100

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 OCTOBER 1999	Date of mailing of the international search report 27 OCT 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RALPH GITOMER Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15957

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RANALDI G. Epithelial Cells in Culture as a Model for the Intestinal Transport of Antimicrobial Agents. Antimicrobial Agents and Chemotherapy, July 1992,Vol.36,No.7 pages 1374-1381.	1-100
Y	HIDALGO I. Carrier Mediated Transport and Efflux Mechanisms in Caco-2 Cells. Advanced Drug Delivery Reviews, 15 November 1996, pages 53-66.	1-100